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CANCER RESEARCH

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CANCER RESEARCH

VOLUME 10 MAY 1950 NUMBER 5

Chemical Studies on the Transformation of Mouse Epidermis to Squamous-Cell Carcinoma: *A Review*

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So many tumors and their normal homologous tissues are used in cancer research today, that the choice is difficult of those most likely to yield a better understanding of the events occurring in the intermediate or precancerous stages. Cowdry's integrated program on epidermal carcinogenesis was initiated about 10 years ago, and, having been associated with this project since its inception, it is the author's opinion that epidermis is more ideal than any other tissue for the study of carcinogenesis for the following reasons: (*a*) Rigid standardization of the experimental conditions allows maximal correlation of chemical, histological, and physical investigations; (*b*) the carcinogen can be applied externally in a uniform manner with fairly uniform results; (*c*) the cells, which are of a single type arranged in layers of increasing differentiation, can be obtained practically free from contamination with cells other than those of epidermal origin; (*d*) the tissue lends itself readily to chemical analysis, for which it is suited because it is avascular, and therefore free from interference by blood; and (*e*) some features in the development of the cancer can be followed with the naked eye from the first application of the carcinogen through the hyperplastic or precancerous stage to the time of first appearance of cancer. It is thus possible to obtain tissue for study at selected stages of cancer development without sacrificing animals needlessly.

The morphological alterations induced in the epidermis by methylcholanthrene are best illustrated with photomicrographs. Normal mouse epidermis (Fig. 1) is composed of two to three layers of undifferentiated cells which respond very quickly to the carcinogen to form many layers with differentiation (Fig. 2). A final stage of carcinogenesis, our transplantable squamous-cell carcinoma No. 1, is shown in Fig. 3. This tumor is a fairly well differentiated, rather fast growing carcinoma, which was originally induced in the skin of a Swiss mouse by the topical application of methylcholanthrene by Cooper, Firminger, and Reller (1). It is now in its 100th generation.

In this paper, reference will be largely limited to a discussion of the chemical alterations produced by methylcholanthrene, since Cowdry (2–5) has described some of the parallel morphologic changes induced in the epidermis by this carcinogen. Since our program was first initiated, methylcholanthrene (0.6 per cent wt/vol) in benzene has been used as the cancer-producing chemical agent. This carcinogen is applied with a camel's-hair brush to a wide area of the shaved back of mice on alternate days, and the animals are sacrificed 5 days after the last application. Before treatment with the carcinogen, the backs of the mice are shaved, and the mice are then allowed to rest for 2 days prior to treatment. The influence of the size of the area, mode and frequency of application of methylcholanthrene, and of other possible experimental variables have been investigated by Cramer and Stowell (6, 7). A procedure for the separation of mouse, rat, and human epidermis from the dermis by placing the whole skin (dermis down) on a warm plate at 50° C. for 1 or 2 minutes, after which the epidermis can be peeled off quite readily, was devised by Baumberger, Suntzeff, and Cowdry (8). Figure 4 shows how completely the epidermis can be separated from the dermis. Also, it has been found possible to separate mouse epidermis

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at room temperature by stretching the whole skin tightly over a sheet of plastic on a wooden block and then separating the epidermis by blunt dissection (9). This procedure was necessary since a temperature of 50° C. was found to have a destructive effect upon the activities of certain enzymes in the epidermis of young mice (55).

Minerals

Since only small amounts of epidermis are available unless large numbers of mice are used, microchemical methods were necessary for the quantita-

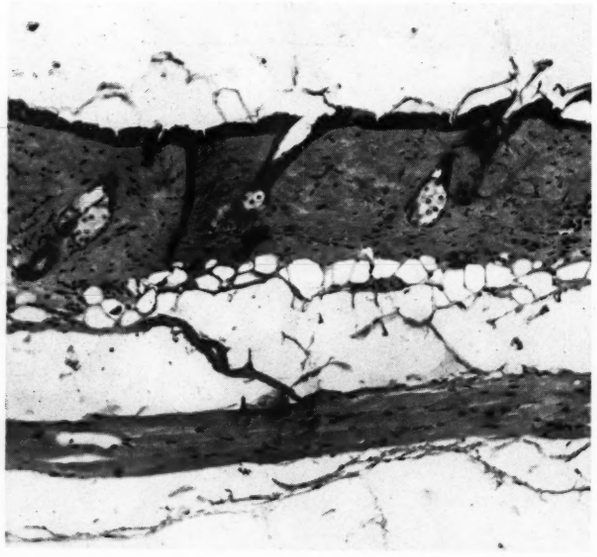

Fig. 1.—Epidermis of an untreated mouse consisting of two layers of undifferentiated epithelial cells with a thin layer of keratin.

tive determination of the mineral content of this tissue and the carcinoma. The levels of magnesium, sodium, zinc, copper, and iron were determined polarographically (10–12). The potassium content was determined by the method of Conzolazio and Talbot (13), while the calcium content was determined by the micro method of Lindner and Kirk (14). Nucleoprotein phosphorus, determined by the method of Berenblum, Chain, and Heatley (15), was employed as the basis of reference in these studies rather than the wet weight of tissue, because of the possibility of loss of water at 50° C., the temperature at which the epidermis was separated. Subsequent work demonstrated, however, that no significant loss of water occurred (9).

Alterations in the mineral content of epidermis undergoing carcinogenesis with methylcholanthrene, and of the analogous transplantable squamous-cell carcinoma, have been represented diagrammatically (12). As early as 10 days after a single application of the carcinogen, the iron:nucleoprotein phosphorus (Fe:NPP) and the calcium-nucleoprotein phosphorus (Ca:NPP) ratios dropped to about 50 per cent of normal epidermis. After prolonged action of methylcholanthrene on the epidermis, there were further slight reductions in the Fe:NPP and Ca:NPP ratios. The copper:NPP ratio was lowered by three applications of the carcinogen during a 10-day period to 45 per cent of normal, and this diminution was maintained essentially the same for 20, 30, and 60 days, at which time the mice had received 6, 12, and 24 methylcholanthrene paintings, respectively. On the other hand, the zinc:NPP ratio was diminished to only 20 per cent of normal after three treatments with the carcinogen, and at 20, 30, and 60 days a decrease to about 30 per cent of normal was maintained. Therefore, zinc and copper appear to occupy an intermediate position as to the extent of de-

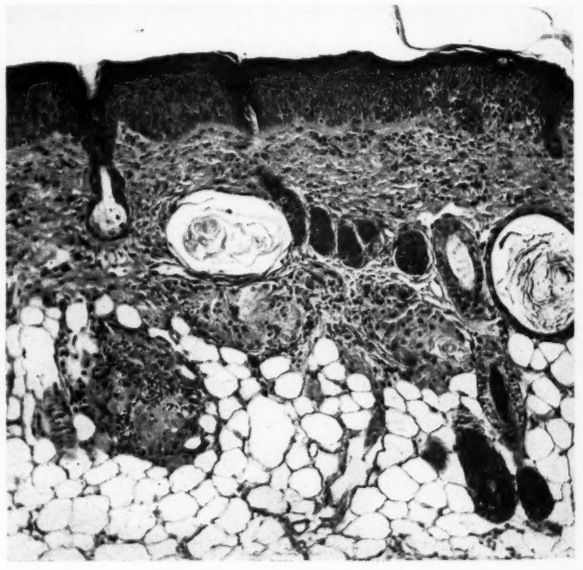

Fig. 2.—Hyperplastic mouse epidermis which is well differentiated containing six to eight layers of cells covered with a thick coat of keratin.

crease when compared with iron and calcium. Sodium, magnesium, potassium, and ascorbic acid were not appreciably altered through the hyperplastic stage.

These studies show that in epidermis rendered hyperplastic in this fashion a new chemical equilibrium is established as early as 10 days after one treatment with the carcinogen and is maintained essentially unchanged until the time when carcinomas usually develop.

In the carcinoma, the levels of zinc, iron, copper, and calcium were very low (12). Thus, the relationship of the inorganic ions studied is different

in the carcinoma from that in hyperplastic epidermis. Since the calcium content of this tumor varied directly with the amount of necrosis (16), only small tumors with little, if any, necrotic tissue were included in the material analyzed.

One of our purposes has been to compare the process of epidermal carcinogenesis of mouse with man. Our first study was to determine the mineral composition of normal human epidermis by the same methods as were used for the mouse. With the limited material at our disposal, no relationship between the mineral content and the age, sex, associated lesion, or the location of the skin was apparent (17). The over-all averages for each metal per 100 milligrams of fresh epidermis were as follows: 0.322 mg. of potassium, 0.122 mg. of sodium, 0.018 mg. of magnesium, and 0.015 mg. of calcium.

A comparison of the role of minerals in epidermal carcinogenesis in mouse and man (18) is shown in Table 1. The calcium content of normal mouse epidermis is nearly 3 times higher than that of normal human epidermis, whereas the copper content of both is approximately the same. The zinc content of mouse epidermis is more than twice that of the human. The calcium content of hyperplastic mouse epidermis is 57 per cent less than normal but is only slightly greater than that of the normal human epidermis. The potassium and sodium contents of the former are 6 and 13 per cent higher, respectively, than those of the latter; while the magnesium content of these two tissues is about the same. Evidently, methylcholanthrene-treated mouse epidermis and normal human epidermis are closely similar insofar as these metals are concerned. They also show analogous histologic appearances. The calcium, zinc, and copper contents of the transplantable squamous-cell carcinoma of the mouse were, respectively, 80, 67, and 83 per cent less than that of untreated mouse epidermis, while in human squamous-cell carcinomas they were, respectively, 47, 30, and 70 per cent less than in normal human epidermis. Hence, there is a close similarity between the transplantable mouse squamous-cell carcinoma and human squamous-cell carcinomas with respect to the contents of calcium, copper, and zinc.

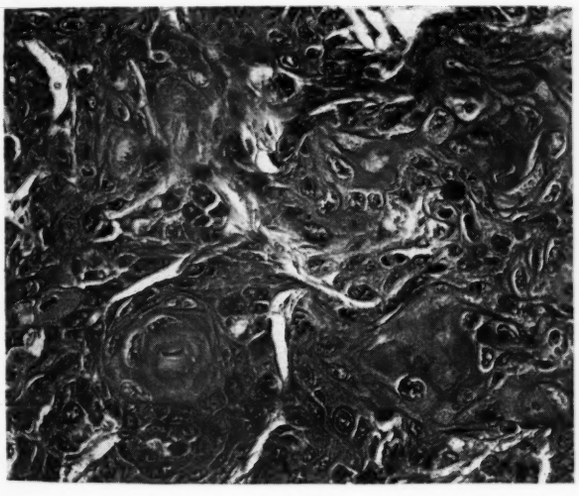

FIG. 3.—Transplantable squamous-cell carcinoma No. 1

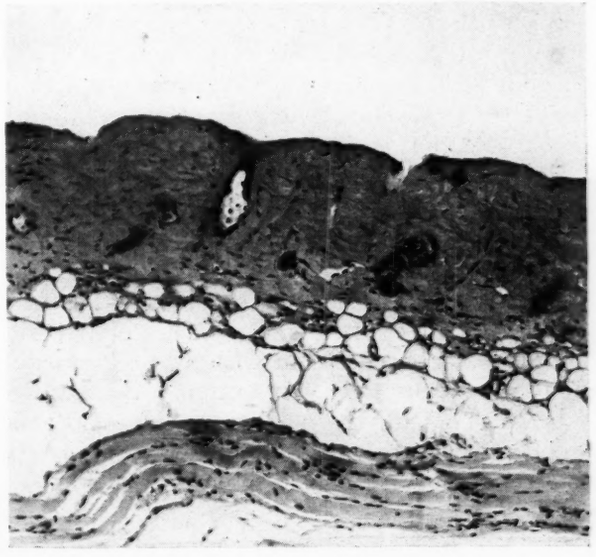

FIG. 4.—Dermis from mouse skin after complete removal of epidermis at 50° C.

The distribution of ultrafilterable and non-ultrafilterable calcium in epidermis, normal and hyperplastic, and in the transplantable carcinoma was determined by Lansing, Rosenthal, and Au (18). In the normal and hyperplastic epidermis the ultrafilterable calcium was 38 per cent of the total calcium, while in the carcinoma the former was reduced to 29 per cent of the latter. In experiments with Ca^{45}, Lansing *et al.* showed that normal

TABLE 1

MINERAL COMPOSITION OF EPIDERMIS

MATERIAL	MOUSE					
	Micrograms of metal per 100 mg. epidermis					
	K	Na	Ca	Mg	Zn	Cu
Normal	347	168	44.0	19.0	5.2	0.58
Benzene-treated	351	163	42.0	19.0	5.5	0.58
Hyperplastic	346	141	19.0	22.6	3.8	0.33
Carcinoma	326	141	9.0	18.0	1.7	0.10
	HUMAN					
Normal	322	123	16.0	18.0	2.4	0.54
Carcinoma			8.5		1.7	0.16

mouse epidermis exchanged this metal actively (19). In early methylcholanthrene-induced hyperplasia there was a loss in storage capacity for calcium. Epidermis in later hyperplasia and in the transplantable squamous-cell carcinoma was completely unable to exchange this metal. This characteristic of the carcinoma is believed to result from an alteration in the calcium-binding complex at the cell surface.

Since calcium gives stability to cell surfaces and to intercellular substances (21), the ease of displacibility of cancer cells by centrifugation may be associated with the decrease in this metal (22). Coman (23) has suggested that the decreased adhesiveness of squamous cancer cells of man, which also have a low calcium content (18), is facilitated by the low level of this metal and thus makes easier the formation of metastases.

However, the decrease in the content of calcium in hyperplastic epidermis may be associated with differentiation rather than with a change specific for precancerous cells. For example, Scarlet Red induces a benign hyperplasia in mouse epidermis, which is accompanied by nearly a 40 per cent drop in the epidermal calcium content (24). Furthermore, new-born mice which have a well differentiated epidermis also have a calcium content similar to that of methylcholanthrene-treated epidermis (24). Finally, normal human epidermis, comparable to hyperplastic mouse epidermis except that the latter does not have sebaceous glands, has a calcium content similar to that of new-born and hyperplastic epidermis. Probably, hyperplastic epidermis serves as a better control for comparative purposes with the carcinoma than does normal epidermis.

The decrease in the calcium content of hyperplastic epidermis is paralleled by a fall in the amount of urea (25). In hyperplastic epidermis the urea level was 55 per cent of the normal epidermis, and in the carcinoma the amount of this constituent was about one-third of the normal epidermis. Furthermore, benign hyperplastic epidermis with sebaceous glands intact, produced by the application of croton oil, had a urea content similar to that of hyperplastic epidermis. This is another example of a change associated with altered cell types and is probably not specific for precancerous epidermis. On the other hand, no change occurred in the ammonia content during carcinogenesis, but the total nitrogen and trichloracetic acid soluble nitrogen decreased in the carcinoma (25).

These studies with calcium in benign and hyperplastic epidermis emphasize the necessity of comparing benign and precancerous conditions as far as is possible to separate chemical alterations due to a change in cell types from those in cells which may actually become malignant. Even under the best conditions, this situation is difficult to evaluate, and changes which occur in later precancerous lesions may give a better hint as to the mechanisms involved in cancer formation. Furthermore, since squamous-cell carcinomas arise in the skin of mice at only one or a few foci (7), it becomes quite obvious that the chemical alterations which might occur in these locations would be difficult to assess when the entire hyperplastic epidermis is used for chemical analysis.

In this connection, the investigations of Berenblum and Shubik on the induction of skin tumors by a single application of 3:4-benzpyrene or 9:10-dimethyl-1, 2-benzanthracene, followed by repeated applications of croton oil are of interest. Further studies revealed that the tumor incidence varied from group to group, but the average latent period was the same when different carcinogens were used and when the croton oil treatment was kept constant (27). When the application of the croton oil was delayed until after the application of the carcinogens, the tumor incidence remained the same, but the latent period varied approximately to the lengths of the intervals free from treatment with croton oil. It was concluded that the initial action in epidermal carcinogenesis was a sudden or irreversible process whereby some normal cells were permanently altered "latent tumor cells" which remained dormant among the hyperplastic cells. If this concept—certainly one difficult to prove or disprove at the present time— is true, chemical analysis of the actual premalignant areas in epidermis would only give data pertinent to the critical changes accompanying malignancy.

Cowdry, Van Dyke, and Geren have shown that the maximum mitotic frequency of the hyperplastic epidermal cells was predominately in the single layer of basal cells in contact with the basal membrane (28). Studies by Cooper and Reller indicated that methylcholanthrene acted first as a stimulant to cell division in the ear epidermis of mice, which was followed by a period of less mitotic activity (29). When carcinomas were about to develop, a marked increase in the mitotic activity in the epidermis occurred again.

Although starvation of mice or the restriction of their diet to 50 per cent of that consumed by controls reduced the mitotic activity of the ear epidermis to low levels (30), the induction of skin papillomata in the interscapular region by a single application of benzpyrene or methylcholanthrene followed by repeated doses of croton oil was independent of the number of epidermal mitoses during the time of action of the carcinogens (31).

Lipids

Wicks and Suntzeff previously demonstrated that the total lipid:protein nitrogen ratio and the cholesterol:protein nitrogen ratio dropped about 50 per cent in hyperplastic epidermis as compared with normal epidermis (32, 33).

That the decrease in total lipid is probably associated with the disappearance of the sebaceous glands is apparent from the studies of Simpson and Cramer (34). These investigators, by employing ultraviolet fluorescence microscopy and studying frozen sections, showed that a regular sequence of events occurred after a single application of methylcholanthrene. Immediately after the application of the latter, the greatest concentration of the carcinogen was seen in the sebaceous glands and in the free lipids of the keratin layer. Then degeneration and disappearance of the sebaceous glands was accompanied by a massive excretion of the carcinogen into the hair follicles, followed by expulsion onto the keratinized surface of the epithelium. The destruction of the sebaceous glands by the carcinogen was complete within 4–5 days following the methylcholanthrene painting. This corresponded to the time required for the depression of the total lipid content to about 50 per cent of the normal. It appears, therefore, that the decrease in the total lipid is associated with the disappearance of these structures. The surface keratin, laden with the sebum containing the carcinogen, was gradually lost by desquamation. There was no evidence that the unchanged carcinogen was taken up directly by the epithelial cells of normal epidermis. Fluorescence microscopy revealed that the unchanged carcinogen disappeared from 6 to 10 days after the application.

Suntzeff *et al.* (35) have found that tumors did not occur in the epidermis of new-born Swiss mice following a single application of methylcholanthrene, whereas by this treatment about 40 per cent of adult mice of the same strain developed carcinomas (36). This lack of response of the epidermis of new-born mice is probably due to the following factors: (*a*) the presence of a well differentiated epidermis in the new-born mouse, composed of seven or more layers of cells covered with a thick coat of keratin; and (*b*) the rudimentary nature or absence of sebaceous glands and hair follicles, the latter being a portal of entry of the carcinogen into the sebaceous glands. It would appear that the carcinogen must first enter the sebaceous glands via the hair follicles before tumors will develop.

Latarjet and Lacassagne (37) discovered that tumors did not develop in the skin of mice by the topical application of methylcholanthrene if the sebaceous glands had been destroyed by previous ultraviolet irradiation. Smith and Rous (38) have also demonstrated that skin of new-born mice of the C strain was very refractory to the same carcinogen which they described as being due to cutaneous conditions which prevented necessary exposure of the carcinogen to the deeper epidermal cells.

Since the sebaceous glands in mice rapidly disappear following the application of methylcholanthrene, the question arises as to whether the disappearance of these structures is prerequisite to the formation of squamous-cell carcinoma in mice as well as in other species. Friedewald and Rous have found that benzpyrene and methylcholanthrene gave rise to sebaceous gland adenomas occasionally in rabbits, while tar caused the disappearance of these structures at an early period after treatment was begun. Yet the benign tumors produced by all three agents were nearly the same in kind (39).

A comparative study of the action of 9,10-dimethyl-1, 2-benzanthracene in the skin of mice, rats, rabbits, and guinea pigs was carried out by Berenblum (40). The order of responsiveness of the epidermis was: rabbit, mouse, rat, and guinea pig, and in these animals this carcinogen was the most potent thus far tested. However, the behavior of the sebaceous glands during carcinogenesis was not mentioned, so the relationship of these structures to epidermal carcinogenesis in the rat, rabbit, and guinea pig needs further clarification.

In an attempt to explain the lack of response of the epidermis of new-born mice to methylcholanthrene, total lipid, lipid phosphorus, and cholesterol were determined in the epidermis of mice of the following ages in days: 2–7, 14, 21, and 60 (41). The above-named constituents were about 50 per cent less in the epidermis of mice from 2 to 7 days of age and in hyperplastic epidermis as compared with normal epidermis, probably because hyperplastic epidermis is devoid of sebaceous glands while in the epidermis of new-born mice these structures are rudimentary. At the age of 14 days a single application of the carcinogen resulted in tumors in some of the mice, and at this age the content of total lipid, lipid phosphorus and cholesterol approached the levels characteristic of epidermis of the adult mouse. It was, therefore, concluded that the response of the epidermis to this carcinogen is correlated with the development of sebaceous glands and possibly with their production of lipids.

According to Glucksmann, the primary reactions of mouse epidermis to benzpyrene were increase in cell growth, a delay in the onset of differ-

entiation (an increase in the phase of the resting cells), and an increase in the number of resting cells (42). These alterations resulted in hyperplasia of the epidermis and its appendages, and in the genesis of papillomas. These changes, according to Glucksmann, are specific, since benzpyrene did not produce such morphological manifestations in the skin of the rat, a nonspecific species, and, furthermore, they were not elicited in the skin of mice by noncarcinogenic hydrocarbons. However, after a long latent period, tumors have been produced in rat skin with benzpyrene (43). In this laboratory, we have found that six applications (3 times weekly on alternate days) of methylcholanthrene (0.6 per cent in benzene) to the shaved skin of rats did not induce hyperplasia (24), yet, after a long latent period, tumors have been induced by this carcinogen (44). More recently, Berenblum has found that 9,10-dimethyl-1, 2-benzanthracene was carcinogenic for rat epidermis (26), and 6, 12 or 24 applications of this carcinogen (0.6 per cent in benzene) applied to the shaved skin of rats on alternate days produced a marked epidermal hyperplasia (24). So it would appear that the specificity or nonspecificity of the epidermis of certain species to carcinogenic hydrocarbons depends upon the potency of the carcinogen, the duration of treatment and upon other factors not necessarily associated with a response to rapid hyperplasia of the epidermis and its appendages. Specific and regional differences in epidermal structure and thickness are probably related to the responsiveness of the epidermis to carcinogenesis (45). In this connection, Twort and Twort have shown that the epidermis of the trunk of mice is more responsive to carcinogenesis than is the lower surface of the foot (46). Heuper has also demonstrated that hairless rats which have more cellular layers and a thicker stratum corneum than their haired litter mates were less susceptible to carcinogenesis by ultraviolet light (47).

Only the sebaceous glands and subcuteaneous fatty tissue of mouse skin exhibited lipase activity, and the activity of this enzyme rapidly disappeared in these locations following treatment of the epidermis with methylcholanthrene (48).

Weigert, Calcutt, and Powell have shown that the only metabolic product of benzpyrene in the skin of mice was the water-soluble BPX_2 (49). The metabolism of this carcinogen started about 1 hour after the application to the skin, and a fairly rapid accumulation of BPX_2 followed. The amount metabolized then gradually diminished.

Recently, evidence was presented for the existence of a qualitative chemical change in carcinogenesis (50). This evidence was uncovered by a difference in the type of polarographic waves and in the half-wave potentials of the reducible material extractable by alcohol and ether from normal and methylcholanthrene-treated mouse epidermis and from induced and transplantable squamous-cell carcinomas. The reducible material from normal and hyperplastic epidermis had a single polarographic wave in buffered solutions at pH 4.4, 5.8, and 7.1, and a double wave at pH 6.3 to 6.4, and at 6.7, while that from the carcinoma had only a single wave at these pH values (51). Moreover, the half-wave potential of the material from epidermis was 200 millivolts more negative at pH 7.1 than that of the carcinoma, and about 100 millivolts more negative at the lower pH values. The diffusion current of the single wave of the material from the carcinoma and of the single and double waves of epidermis was proportional to the concentration of the reducible material. The polarographic characteristics of the substance in the epidermis are changed to that of the carcinoma, as soon as the carcinoma appears, by induction by methylcholanthrene and are the same in transplantable squamous-cell carcinomas of different degrees of anaplasia. Normal human epidermis contains a substance indistinguishable polarographically from that of the mouse, and it also undergoes alteration in squamous-cell carcinomas of man.

TABLE 2

SUMMARY OF CONTENT OF FIVE VITAMIN B FACTORS IN MOUSE TISSUES DURING METHYLCHOLANTHRENE CARCINOGENESIS*

The values are expressed in micrograms per gram of dried tissue, and as per cent of the values for normal epidermis.

B FACTOR	NORMAL EPIDERMIS	BENZENE-TREATED EPIDERMIS		METHYLCHOLANTHRENE-TREATED EPIDERMIS		TRANSPLANTED CARCINOMAS	
	(γ per gm.)	(γ per gm.)	(per cent)	(γ per gm.)	(per cent)	(γ per gm.)	(per cent)
Biotin	0.196	0.194	99	0.125	64	0.149	76
Choline	2471	2802	113	2651	107	6240	252
Inositol	526	568	108	547	104	1154	219
p-Aminobenzoic acid	2.40	2.30	96	2.40	100	3.09	129
B_6 complex	2.45	2.88	118	3.05	124	4.10	167

* From Ritchey *et al.* (52).

VITAMINS

Ritchey, Wicks, and Tatum have determined the quantities of pyridoxine, choline, inositol, *p*-aminobenzoic acid, vitamin B_6 complex, and biotin in the epidermis of mice undergoing carcinogenesis by assay with mutant strains of Neurospora (52). The results, summarized in Table 2, are expressed in μg. of vitamin per gram of dry epidermis. All the vitamins studied, except biotin, increased in the tumors, as compared with the normal and treated epidermis. Choline showed little change in the benzene-treated and hyperplastic epidermis, but rose to 2.5 times greater than normal in the tumor. Likewise, inositol, little affected in the treated epidermis, was 219 per cent greater in the tumor than in hyperplastic epidermis. *p*-Aminobenzoic acid showed no change with benzene alone or with the carcinogen, but was slightly raised in the tumor. There was also a gradual rise in the level of vitamin B_6 complex from the normal to the percancerous stage to the carcinoma.

Only biotin decreased in the hyperplastic epidermis to about 64 per cent of that of normal epidermis.

The increase in choline, as a constituent of lecithin, may be related to the increased phospholipid content of mouse squamous-cell carcinomas (53). The high level of inositol of these tumors may also be correlated with phospholipid if inositol is present in tumors in a form such as was found by Folch (54) in brain cephalin.

ENZYME ACTIVITIES

The activities of succinic dehydrogenase, cytochrome oxidase (55), and apyrase (56) were determined by the methods of Schneider and Potter (57) and Dubois and Potter (58), respectively. Arginase activity (25) was determined by the procedure of Roberts (59) and the level of cytochrome c (60) was determined polarographically (61) and spectrophotometrically. The results are best summarized in Figure 5 where the activities of succinoxidase and cytochrome oxidase are expressed in the conventional manner as Q_{O_2}; cytochrome c as μg. per 100 mg. fresh tissue; apyrase as the μg. P liberated per mg. fresh tissue per 15 minutes; and arginase as the μg. urea liberated per mg. fresh tissue under the standard assay conditions (59).

The apyrase and succinic dehydrogenase activities of normal epidermis were low and remained unchanged in methylcholanthrene-treated epidermis, but the activities of both enzymes were increased significantly in the transplantable carcinoma. The level of cytochrome c also was constant throughout the hyperplastic state but decreased in the carcinoma. Arginase activity was fairly constant in the precancerous stages as compared with normal epidermis, except in the epidermis which received three paintings of the carcinogen in which tissue the arginase activity was 3 times greater (not shown in chart) than the normal. In tumor No. 1 the activity of this enzyme was 6 times greater than normal epidermis, and in tumor No. 2, a more highly differentiated carcinoma, the activity was

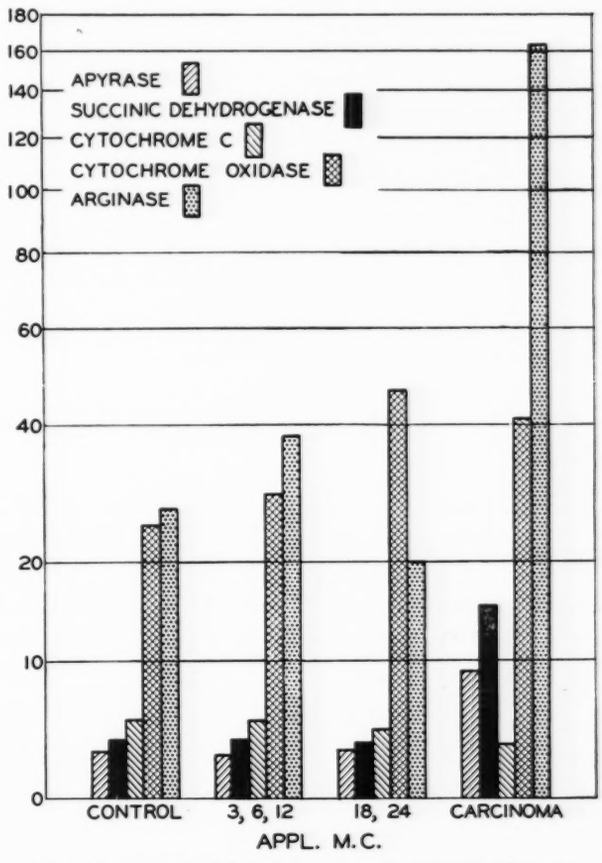

FIG. 5.—The activities of apyrase, succinic dehydrogenase, cytochrome oxidase, and arginase and the level of cytochrome c in epidermal carcinogenesis. The results are expressed as described in the text.

18 times (not shown in chart) that of normal epidermis.

The activity of cytochrome oxidase in normal epidermis was much lower than most normal tissues (62) and was practically unchanged in early hyperplastic epidermis (3, 6, and 12 treatments with the carcinogen). However, in the epidermis which had received 18 and 24 treatments with the carcinogen the activity of this enzyme was nearly twice that of the normal epidermis. This is the only constituent that has been found to increase significantly in the late hyperplastic epidermis prior to the formation of cancer. The activity of this enzyme dropped slightly in the latter tissue.

Although the activity of succinic dehydrogenase, apyrase and cytochrome oxidase are low in normal epidermis, their activities approach that found in tumors derived from other tissues (62, 63) and are in agreement with the concept of Greenstein in that "a cancerous tissue can be described by a chemical pattern which is largely similar to that of nearly all other cancerous tissues regardless of their etiology, histogenesis, or even species wherein found" (63).

Although calcium may be associated with the activity of the succinic dehydrogenase and apyrase systems (64) and iron and/or copper with the cytochrome oxidase system (65), no relationship appears to exist between the activities of these enzymes and the decrease in calcium content in hyperplastic epidermis.

Amino Acids

Normal and hyperplastic epidermis and the transplantable carcinoma were assayed by microbiological methods for lysine, isoleucine, leucine, methionine, valine, phenylalanine, threonine, histidine, glutamic acid, cystine, arginine, and tryptophane by Roberts *et al.* (66). The sum of the amino acids showed highly significant increases over the normal in hyperplastic epidermis and in the carcinoma, whether expressed on a dry weight or on an amino acid nitrogen basis per 100 mg. tissue. Benzene, the solvent for the carcinogen, produced changes in the distribution of amino acids in the epidermis which was different from that of the carcinogen in benzene in that the cystine content was higher and the methionine content lower than that of hyperplastic epidermis. However, the distribution of the amino acids in the carcinoma was quite similar to that of the precancerous epidermis, and, therefore, showed no distinct pattern characteristically different from nonmalignant tissue.

These results again emphasized the fact that early and late hyperplastic epidermis, in which the cells are in general the same, morphologically and chemically, serves as a better basis of reference for the carcinoma than does normal epidermis. Furthermore, the advantage of this comparison allows the investigator to discover data which may be more obviously pertinent to the nature of the maligant transformation of cells.

Roberts and Tishkoff (67) demonstrated by paper partition chromatography that hyperplastic epidermis and epidermis of new-born mice, both of which are quite similar with respect to histological appearance and rate of growth, have larger amounts of free amino acids than the normal epidermis of mice. However, the carcinoma showed an over-all decrease in content of free amino acids. These results suggested to the authors "that the growth in the nonmaligant epidermis may be associated with the ability of cells to increase the intracellular concentration of the amino acids necessary for protein synthesis, whereas in malignant tumor the mechanisms for protein synthesis are much more efficient and can operate at a greatly accelerated rate, even in the presence of smaller concentrations of amino acids." In line with this concept, Zamecnik *et al.* (68) have found that the rate of incorporation of C^{14} carboxyl-labeled DL-alanine into surviving slices of *p*-dimethylaminoazobenzene-induced hepatomas was 7 times that of slices of control livers and 2.5 times that of nonmalignant portions of the liver. This incorporation was accounted for in the alanine fraction of the liver proteins. These experiments demonstrated that hepatoma cells incorporated alanine into protein more rapidly than did the normal liver cells, probably via a greater rate of peptide bond synthesis.

With paper chromatography a consistant pattern of free amino acid distribution was found in squamous-cell carcinomas and in some other malignant tissues, while most normal tissues differed from each other in this respect (69). This similarity of tumors is consistent with the like pattern in enzyme and vitamin composition of some neoplastic growths (63, 70).

DISCUSSION AND SUMMARY

The advantages of epidermis of mice for the study of carcinogenesis have been briefly described. Since the epidermis of other species responds to the potent carcinogen, 9, 10-dimethyl-1, 2-benzanthracene (40), a comparison of the carcinogenic process in a variety of animals is possible. Many constituents of this tissue in the mouse have been quantitatively determined to ascertain the mechanism by which this tissue becomes carcinomatous. Of the minerals studied, the levels of iron, copper, zinc, and calcium were less in hyperplastic epidermis and in the carcinoma than in normal epidermis, but some experiments suggest that the drop in calcium, and perhaps other constituents, may be associated with altered cell types rather than with a change specific for pre-malignant epidermis. Investigations with radiocalcium suggest an alteration in the calcium binding complex at the cell surface of the transplantable squamous-cell carcinoma. Coman's studies indicate that the decreased adhesiveness of squamous carcinoma cells, which have a low calcium content, may play a role in the formation of metastasis.

The sebaceous glands appear to be important structures in the response of mice to methylcholanthrene, since the skin of new-born mice in which these structures are rudimentary do not respond to this carcinogen. Furthermore, adult skin treated with this carcinogen loses its sebaceous glands concomitantly with a drop in the content of phospholipid, total lipid, and cholesterol. Repeated application of the carcinogen leaves the epidermal cells without secretions from these glands and in a new chemical environment. Whether the disappearance of the sebaceous glands in other species is a prerequisite to cancer formation has not yet been decisively answered. In the total lipid of the epidermis during carcinogenesis there is a lipid-like substance which undergoes alteration at the onset of malignancy.

Of the water-soluble vitamins studied, pyridoxine, choline, inositol, and *p*-aminobenzoic acid increased in the carcinoma, while the biotin and ascorbic acid levels were not appreciably changed from that in epidermis. The rise in the level of choline and inositol may be associated with the increased amount of phospholipid in the tumor.

Mouse epidermis is characterized by a low activity of succinic dehydrogenase, cytochrome oxidase, and apyrase. In late hyperplastic epidermis only cytochrome oxidase is increased significantly, and this is the only cellular component found to date which changes prior to malignancy. In the carcinoma, the activity of apyrase, succinic dehydrogenase, and arginase are markedly increased, while that of cytochrome oxidase and cytochrome c are decreased.

The assay of twelve amino acids in the epidermis undergoing carcinogenesis revealed that their distribution in the carcinoma was quite similar to that found in hyperplastic epidermis, but the sum of the amino acids in these two stages of carcinogenesis was significantly increased over that of normal epidermis. Hyperplastic epidermis had larger amounts of free amino acids than did normal epidermis, while the carcinoma showed an over-all decrease in these constituents.

A survey of the many constituents determined in the epidermis of mice undergoing carcinogenesis reveals some very interesting changes from normal epidermis to the hyperplastic state and from the latter to the carcinoma. However, in hyperplastic epidermis it has been found that with but one exception, cytochrome oxidase in late hyperplasia, all the components change abruptly from the normal to and throughout the precancerous epidermis and abruptly from the latter to the carcinoma. As has been pointed out previously, all chemical analysis of hyperplastic epidermis represents pooled samples of large areas of treated epidermis, so that chemical alterations arising in the few actual pre-malignant areas are diluted with cells of the former. Only when procedures become available for ascertaining the actual epidermal foci which will give rise to carcinomas will it be possible to determine chemical alterations critical to the problem of neoplasia of epidermis. So even in a tissue such as epidermis, in which the experimental conditions for carcinogenesis can be rigidly controlled, assessment of chemical changes due to alterations in cell types from those characteristics of real pre-malignancy are most difficult to evaluate. Such conditions must certainly exist to an even greater degree in other organs or tissues where criteria for the pre-malignant state are even more difficult to establish and assess.

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Some Effects of 2-Acetylaminofluorene on the Dog*

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The compound, 2-acetylaminofluorene, was recognized as a carcinogen in 1941 by Wilson, DeEds, and Cox (15). This compound, incorporated in the diet in amounts varying from 0.03 to 0.15 per cent, will elicit the formation of tumors in various tissues of the rat, mouse, and cat (12, 3, 5). The hepatoma is one of the most common neoplasms produced by this drug in the rat. The purposes of the following experiments were to determine the carcinogenic activity of 2-acetylaminofluorene in the dog and to discover some of the changes in metabolism produced by the drug, particularly those associated with the induction of hepatomas. Changes in liver function and structure are revealed in the dog by an alteration in the urinary uric acid/allantoin ratio. If the liver cells are sufficiently destroyed or altered in character, the ratio increases reflecting a reduction in the activity of uricase (2, 6). This ratio was used, therefore, in the experiments described herein, to detect changes in liver function. Depletion in liver riboflavin and liver protein has been associated with the induction of hepatomas by carcinogens in the rat (7–11). A study of the effects of 2-acetylaminofluorene on the utilization of riboflavin and proteins by dogs was included, therefore, in the following experiments.

MATERIAL AND METHODS

The drug 2-acetylaminofluorene was incorporated (0.03 per cent) in the synthetic diet (Table 1) which was fed to twelve adult, male dogs (6 beagles and 6 mongrels, 2–6 years of age). Nine male control animals (3 mongrels and 6 beagles, 2–6 years of age) were fed the diet without the drug.

The technic of Najjar and Holt (14) was used to determine retention of riboflavin in dogs. For this determination, 0.02 mg. of riboflavin/kilogram of body weight was injected, and the quantity of the vitamin excreted in the urine (13) was measured over periods of 4 and 8 hours. The riboflavin content of the liver and feces were determined by the method of Arnold (4).

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RESULTS AND DISCUSSION

All the dogs fed the 2-acetylaminofluorene developed evidences of depletion in protein stores and in riboflavin. After 6 months of feeding, the urinary nitrogen excretion on a protein-free diet, the albumin/globulin ratio, hemoglobin content of the blood, and liver nitrogen were reduced to low levels reflecting a decrease in the protein stores. Data on six of the drug-fed animals and on six of the control animals are recorded in Table 2. The nitrogen balance index of the dietary casein also decreased significantly below control values during the feeding period of 6 months. The nitrogen balance index is a function of the retention of dietary nitrogen to build body protein stores, the index increasing as more nitrogen is utilized for this purpose. The decrease in index, therefore, indicates a reduction in the ability of the animals to retain dietary nitrogen in the body. With simple protein depletion there is an increase in the index rather than a decrease. The index for casein, for example, in a protein-depleted dog, will increase to values as high as 0.98 (1). Thus 2-acetylaminofluorene not only depletes the animal in body protein but also leads to reduced retention of dietary nitrogen.

The dogs fed the carcinogenic diet developed a seborrheic erythema with alopecia which is similar to skin conditions associated with riboflavin deficiency. A large excess of riboflavin in the diet of one of these dogs resulted in an improved condition of the lesions. Removal of the excess vitamin from the diet resulted in the reappearance of the lesions. Riboflavin retention tests were conducted on a number of the animals having lesions and on controls. The results of these tests are illustrated in Figure 1. The excretion of the vitamin was less in those animals with the lesions, demonstrating a greater retention of the riboflavin.

A reduction in the content of riboflavin in the liver was associated with the deficiency (see Table 2). The effect on this vitamin is similar to other carcinogens such as *p*-dimethylaminoazobenzene, and it is possible, as suggested by Miller (11), that riboflavin is involved in detoxication processes. It is interesting to note that the excretion of riboflavin in the feces is even reduced when the drug

is added to the diet. This reduction in riboflavin is being correlated with a decrease in the bacterial count of the feces, a decrease which is being studied further in detail.

One of the dogs, examined after approximately 6 months of receiving the carcinogen, had developed a tumor mass on the liver (see Fig. 4). It was believed that hepatomas such as this would increase the urinary uric acid/allantoin ratio. The ratio of excretion of uric acid to that of allantoin has been used in these laboratories for a number of years to detect liver damage in dogs, the ratio increasing as the severity of the damage increases. The data plotted in Figure 2 illustrate the effect of feeding the drug on the ratio. The two dotted lines drawn parallel to the X axis denote the upper and lower limits of the ratio in control dogs fed the diet (0.15 gm. casein nitrogen/kg body weight) without the carcinogen. A marked increase of the uric acid/allantoin ratio to 0.6 or more, illustrated by the large circles in Figure 2, was associated with abnormal proliferation of liver tissue and the development of neoplasms. Grossly, the livers of these animals showed abnormal growths, ranging

TABLE 1

COMPOSITION OF THE SYNTHETIC DIET

	Per kilogram body weight			Mg. per kilogram body weight (per day)
	Calories	Grams		
Casein (Labco)	3.75	1.10	Thiamin	0.03
Sucrose	12.25	3.08	Riboflavin	0.025
Dextrin	13.04	3.26	Nicotinic acid	0.250
Dextrose	26.96	6.74	Calcium pantothenate	0.200
Lard	24.00	2.67	Pyridoxine	0.015
Salt*		0.30	Choline	15.0
Agar		0.47	2-Methyl-1,4-naphthoquinone	0.00001
			Calcium tocopherol monosuccinate	0.5
			Biotin	0.01
			Folic Acid	0.008
			Vitamin A	470 units
			Vitamin D	85 units
TOTAL:	80.00	17.62†		

* Wesson's Modified Salt Mixture.

† Mix 1.4 gm. of water with every gm.

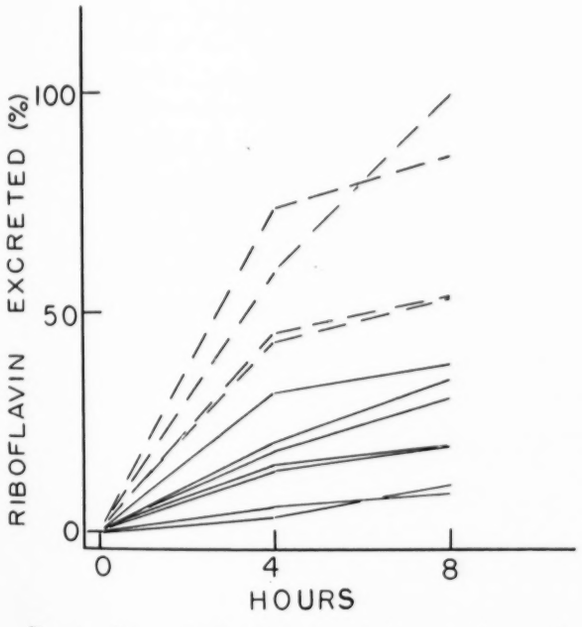


FIG. 1.—Per cent riboflavin excreted versus hours after injection of 0.02 mg. of the vitamin/kg of body weight. The dotted lines illustrate data obtained from control animals; the solid lines, data obtained from animals fed the carcinogen.

TABLE 2

THE EFFECTS OF 2-ACETYLAMINOFLUORENE ON NITROGEN AND RIBOFLAVIN RETENTION

Effects of feeding 2-acetylaminofluorene (AAF) for 6 months in the casein diet (0.15 gm. N/day/kg body weight) on the protein stores, on dietary nitrogen retention (Nitrogen Balance Indexes), and on riboflavin. All values are averages obtained on six dogs except those marked with an asterisk which were obtained on four animals. The dogs were given nitrogen-free diets before testing for urinary nitrogen.

Variable		Control	AAF
Albumin			
Globulin		1.2	0.53
Hemoglobin	gm per cent	14.0	8.3
Liver protein	gm/100 gm tissue	17.6±0.03	14.1±0.9†
Urinary N (Protein-Free Diet)	gm/day/sq. m. body surface	2.12*	1.29
Nitrogen Balance Index		0.78*	0.66
Liver Riboflavin	μg/gm tissue	33.3±2.9	15.1±1.7
Feces Riboflavin	μg/day/kg of body wt.	44.8±4.9	21.0±2.9

* Values obtained on four animals.

† Standard error.

from pin-point nodules and cysts to well developed tumor masses. The small circles illustrate data obtained on dogs in which there were no marked gross lesions on the liver.

The gross and microscopic appearances of the livers of the dogs used to obtain the data in Figure 2 are summarized in Table 3 and illustrated in part in Figures 3 to 10.

Dog No. 1 (white circles in Fig. 2) developed multiple nodules and cystic areas in the liver (see Fig. 3) after 30 weeks on the carcinogenic diet. The microscopic appearance of this liver illustrated in Figures 6 and 7 can be compared with the normal liver in Figure 5. The abnormal growth (hepatoma) and surrounding tissues seen in Figures 6 and 7 can be described as follows: The cell size in the hepatoma was normal or reduced, with markedly granular cytoplasm which stained heavily with eosin. The nuclei varied in size—some were hyperchromatic, others pyknotic—but mitoses were not evident. Degenerate cells, signet ring in appearance, were present. The margin of the hepatoma was adjacent to normal liver cells at its entire circumference. Areas adjacent to central veins and the portal triad were more heavily stained than others, and the cells appeared normal. In between, however, fatty infiltration and fatty degeneration were marked. Slight fibrous infiltration was evident.

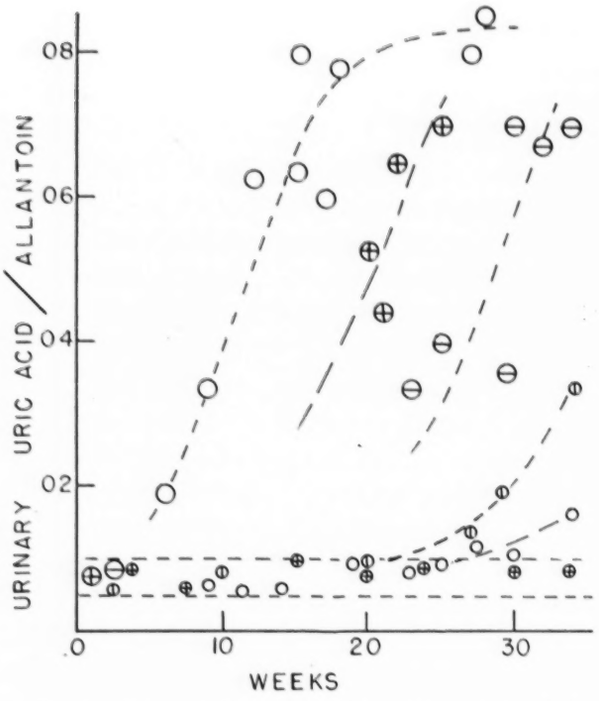


FIG. 2.—Urinary uric acid-allantoin ratio versus weeks dogs were fed diet containing 0.03 per cent 2-acetylaminofluorene. The horizontal dotted lines denote limits in normal dogs. The symbols associated with dog numbers (in Table 3) are: 1, ○; 2, ⊕; 3, ⊖; 4, ⊜; 5, ⊙; ⊛6, .

Dog No. 2 (white circles with crossed bars in Fig. 2) developed a tumor mass, as well as nodules and cysts, after 25 weeks on the carcinogenic diet (see Fig. 4). The microscopic appearance of this liver is illustrated in Figures 8 to 10. There was a complete loss of general liver topography, with a marked increase in fibrous connective tissues (cirrhosis), which had grown completely around liver areas. Fatty degeneration was almost absent. In the tumor, the liver cells were very compact and deeply stained with reduced cytoplasm. Mitotic figures were numerous. A definite cholangioma had developed. The tall columnar cells were in tubular or acinar arrangement, and numerous cross sections were observed.

The liver of dog No. 3 (circles with horizontal bar) developed a tumor mass at the periphery of one of the lobes after 34 weeks on the diet. There

TABLE 3

THE EFFECTS OF FEEDING 2-ACETYLAMINOFLUORENE ON THE GROSS AND MICROSCOPIC APPEARANCE OF THE LIVER IN DOGS

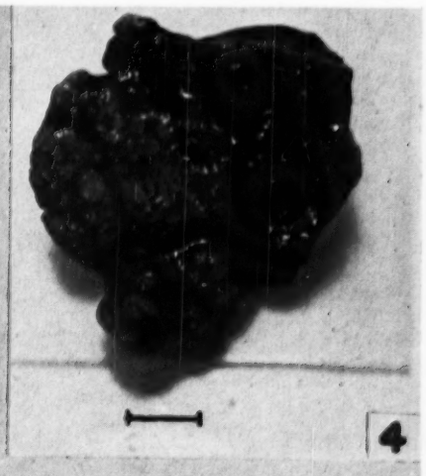
Dog no.	Weeks on diet	Weight, kg.		Appearance of liver	
		start	end	Gross	Microscopic
1	30	10.1	9.0	multiple hard nodules and cysts	hepatoma, varied size cells, some cystic degeneration
2	25	9.8	8.2	tumor mass, multiple nodules, and cysts	cholangioma, glandular and cyst formation cirrhosis with general loss of liver topography
3	34	10.2	9.8	tumor mass at periphery and cysts	fatty degeneration, necrosis, cyst formation (no sections of tumors)
4	34	12.6	14.6	no gross pathology	
5	36	7.4	4.3	no gross pathology	cytoplasmic depleted cells, enlarged sinusoids
6	36	7.5	9.2	no gross pathology	normal

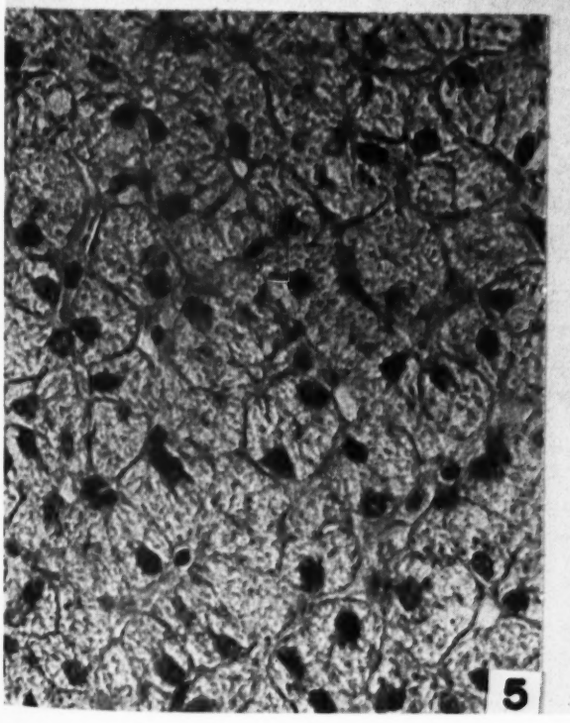
were also numerous small cysts. Unfortunately, sections of the tumor mass were missed, but a microscopic examination of the liver as a whole showed degenerative changes, necrosis, and cyst formation.

Thus, gross and microscopic examinations of the liver demonstrate that the feeding of 2-acetylaminofluorene in the diet can induce neoplasms in the dog. In all dogs examined, however, whether or not tumor masses developed, there was a depletion in protein and riboflavin stores of the body. It may be that the drug reduces the activity of some enzyme systems, possibly associated with riboflavin, creating an enzymatic environment more favorable for the growth of the cancer cell. Whether or not this growth takes place would be a function, in part, therefore, of the type of cellular environment produced. This environment is under investigation.

SUMMARY

1. The carcinogen, 2-acetylaminofluorene, fed in a synthetic diet over a period of 6 months to dogs, caused a reduction in the protein stores of





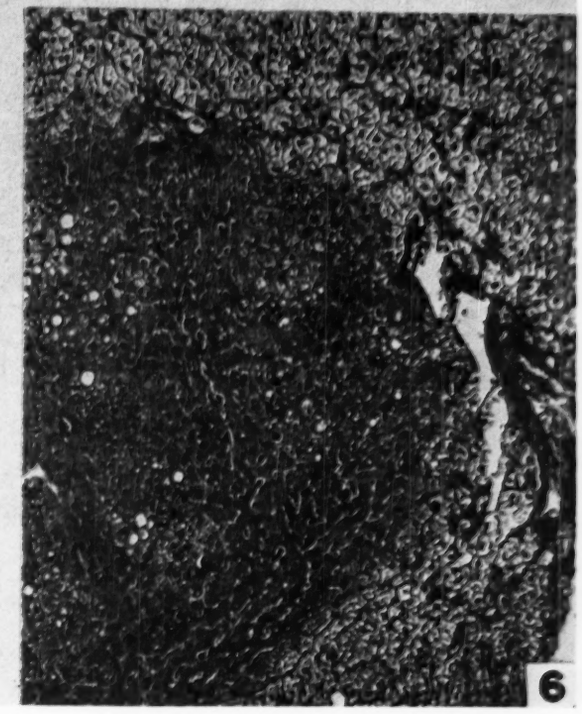
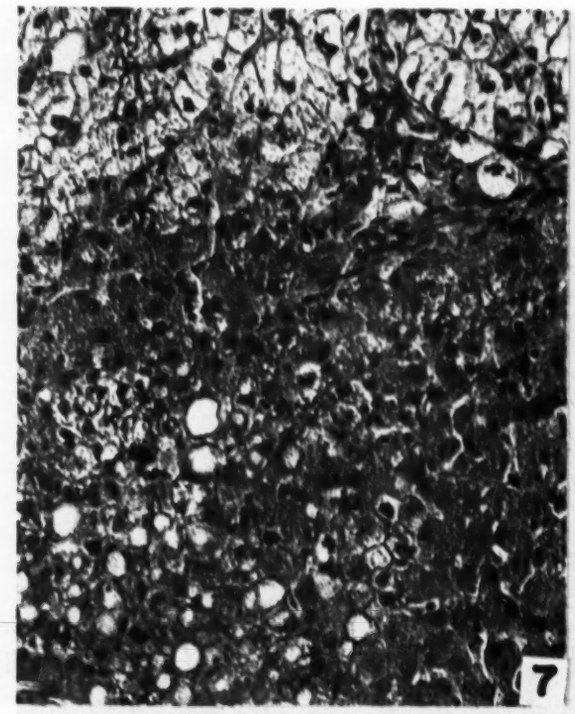


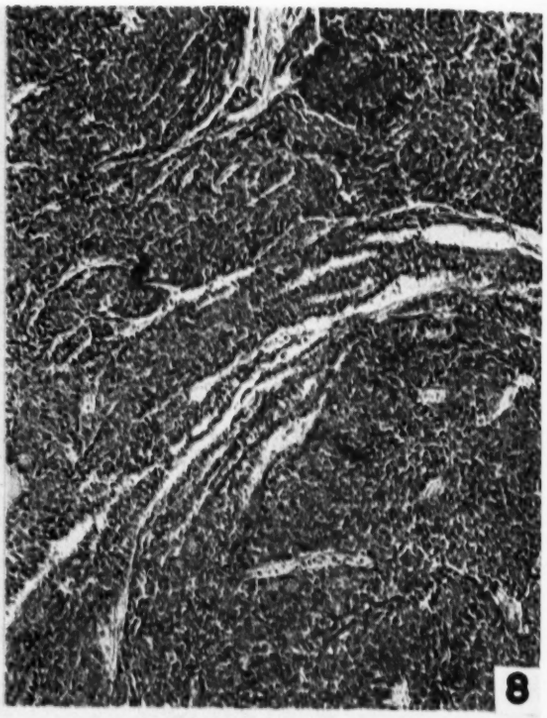
FIG. 3.—Gross appearance of liver of dog No. 1 (see Table 3), the line equals 2 cm.

FIG. 4.—Gross appearance of liver of dog No. 2, the line equals 2 cm.

FIG. 5.—Liver of normal dog. ×560.

FIG. 6.—Liver of dog No. 1 fed 2-acetylaminofluorene for 30 weeks. Hepatoma bordering on normal liver tissue. ×110.





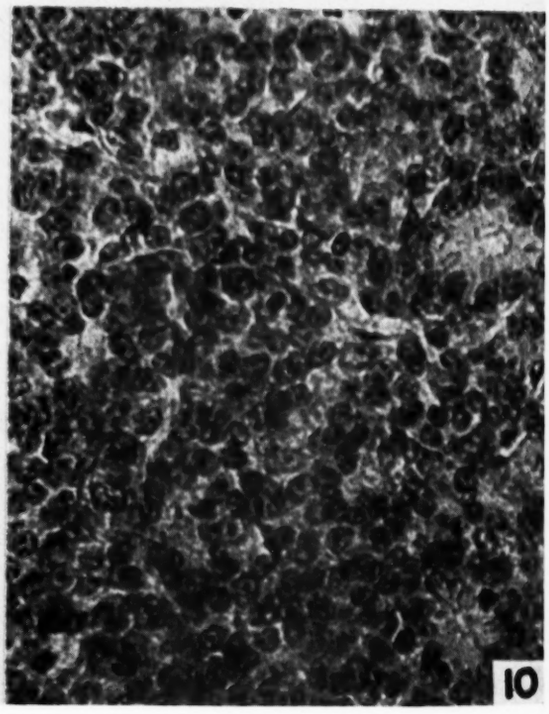


FIG. 7.—Liver of dog No. 1 fed 2-acetylaminofluorene for 30 weeks. Hepatoma. Cell cytoplasm is very granular. Cyst formation. ×330.

FIG. 8.—Liver of dog No. 2 fed 2-acetylaminofluorene for 25 weeks. General loss of liver topography. ×110.

FIG. 9.—Liver of dog No. 2. Fibrosis and liver cell degeneration. ×110.

FIG. 10.—Liver of dog No. 2. Gland formation and packing of cells. ×560.

the body. The plasma albumin/globulin ratio, hemoglobin, red blood cells, liver nitrogen, and protein-free urinary nitrogen all decreased over the feeding period—variables which also decrease when animals are fed a protein-free diet.

2. The retention of dietary nitrogen, as measured by the nitrogen balance index also decreased below control values, demonstrating that the depletion in protein stores was the result of a reduction of retention of both dietary and body nitrogen.

3. A riboflavin depletion was produced in dogs fed 2-acetylaminofluorene. This depletion resulted in the appearance of typical skin lesions, in the reduction of liver riboflavin, and in an increase in the ability of the animal to retain excess injected vitamin. The carcinogen also reduced the excretion of riboflavin in the feces.

4. Hepatic neoplasms developed in three of the six dogs fed the carcinogen from 6 to 9 months. The urinary uric acid/allantoin ratio increased markedly in these dogs, an increase which was associated with the alteration in liver function.

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Effects of Increased Pressure upon Sarcoma 180*

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The clinical behavior of neoplasms frequently suggests a relationship between pressure and tumor growth. For example, the increase in the rate of tumor growth, after the constriction of a capsule or another structure has been removed by biopsy or spontaneous perforation, is familiar to every surgeon. A scirrhous carcinoma of the breast, enmeshed and compressed by dense fibrous tissue, is notoriously slow-growing; yet, these same cells, or their daughter cells, will grow rapidly once they escape to the relatively loose tissue of the regional lymph nodes. Keloids, too, may not appear in regions subject to pressure, such as is exerted by a hat or collar. Numerous other examples will suggest themselves—all susceptible of an interpretation indicating that local pressure or constriction will hinder tumor growth.

The reverse of this relationship—that decreased local pressure tends to initiate neoplastic growth—has been suggested, and the overgrowth in amputation neuromas has been considered a local manifestation of such a "release phenomenon"(2).

There has been little experimental investigation of this problem. Basset (3) has shown that, *in vitro*, mouse Sarcoma 37, suspended in physiological saline, can survive a pressure of 1,000 atmospheres for ½ hour. Other investigators have been concerned with the effects of high or low oxygen concentrations upon tumor growth and have used increased pressures only incidentally, as a means of increasing oxygen tension. Pressure, itself, was considered to be without effect. Thus, de Almeida (1) exposed Roffo spindle-cell rat sarcoma *in vivo* to six atmospheres of oxygen for ½ hour. After 10 days the tumor was hemorrhagic, and tumor cells were destroyed. Campbell (4) could not confirm these results, using Walker rat carcino-sarcoma 256, Bashford mouse carcinoma 63, Twort mouse carcinoma, or spontaneous mammary carcinoma of mice. His rats would tolerate only five atmospheres for 1 hour; the mice only four atmospheres for 1 hour. Campbell and Cramer (5) found that at total pressures of one atmosphere, 60 per cent of an atmosphere partial pressure of oxygen for 2 weeks, caused no inhibition of tumor growth. On the contrary, they, and Warburg *et al.* (11), found that oxygen starvation had some temporary effect in inhibiting tumor growth, and sometimes produced tumor necrosis. Campbell (4) concluded that variations in the partial pressure of oxygen were of no value, by themselves, in tumor therapy. Similar conclusions were reached by Pollack *et al.* (8), who used air at 15- and 30-pound-pressure and subcutaneous injections of oxygen.

Marsh (7) used compressed air at a gauge pressure of 30 pounds. This yielded the same partial pressure of oxygen as Campbell and Cramer used, although the total atmospheric pressure was tripled. He found that the incidence of spontaneous tumors in a tumor-prone strain of albino mice was slightly reduced, and the longevity and tumor age were increased.

This paper presents some results of compression of Sarcoma 180. Two main types of compression were studied. Local compression of the tumor was produced by implanting it into the tail of mice where its growth would lead to compression by the dense skin overlying this region. In another series of mice, the entire tumor-bearing mouse was compressed by increased atmospheric pressure in a compression chamber.

EXPERIMENTAL

Compression by tail implantations.—Sarcoma 180 was implanted into the ventral or dorsal surface of the tail in 58 albino mice of the Rockland Farms strain, using small pieces of tumor, each weighing about 2 mg. A small trocar was used for implantation. Each tumor was transplanted into approximately ten mice. Six experiments were done, summarized in Table 1. No difference was noted between ventral and dorsal implantations.

In all tail tumors growth was extremely slow, and the tumors never became as large as the controls implanted in the usual subcutaneous axillary region (Fig. 1). In five mice the tumors did not take. Of the remainder, 35 became hemorrhagic, the discoloration being clearly visible through the skin of the tail. In 38 mice, the tail tumors regressed completely. (This constitutes 72

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per cent regressions, as compared with a regression rate of 6 per cent in over 1,000 Sarcoma 180 tumors implanted in the usual site and observed in the last 18 months.) Twenty-one of the 58 mice died of the original tumors, from metastases or extensions of the implanted tumors, from a spontaneous and apparently unrelated tumor in one case, and from all other causes. In some cases death occurred from metastases, even though the original tumors had regressed. All the survivors remained free of Sarcoma 180. In two cases the tail was sloughed off distal to the implantation site. The remaining mice showed no evidence of impairment of circulation by growth of the tumor.

Figure 1 illustrates the growth of the tumors in the tail (Table 1, Exp. 3). Figure 2 is a photograph of a tumor 16 days after tail implantation. The tumor was markedly hemorrhagic and regressed completely after 41 days (Fig. 3).

Compression by increased atmospheric pressure.—In another type of experiment the entire tumorous mouse was compressed by increased atmospheric pressure in a pressure chamber. Three such chambers were used in these experiments. A low pressure chamber was made of brass pipe 4 in. in diameter. One end was closed by a screw cap, fitted with a flanged and beveled lucite window, bedded in sealing compound. An insulated wire leading to an internal electric light was introduced through the cap. The other end of the pipe was closed by a blank cast-iron flange held on by bolts and sealed by a rubber gasket. The mice were inserted at the flange end. This chamber was used for pressures up to 110 pounds per square inch. All pressures given in this paper are gauge pressures; total pressures are 15 pounds higher.

Figures 4 and 5 show an improved chamber used for higher pressures. This was made of a seamless

TABLE 1

TAIL IMPLANTATIONS—SARCOMA 180

Exp. No.	Number of tumors implanted (number of mice)	Number of takes	Number becoming hemorrhagic	Number of tumor regressions	Death from all causes
1	9	9	8	7	3
2	10	7	5	6	2
3	10	9	5	9	1
4	9	8	3	6	2
5	10	10	5	5	8
6	10	10	9	5	5
Totals	58	53/58 (91 per cent)	35/53 (66 per cent)	38/53 (72 per cent)	21/58 (36 per cent)

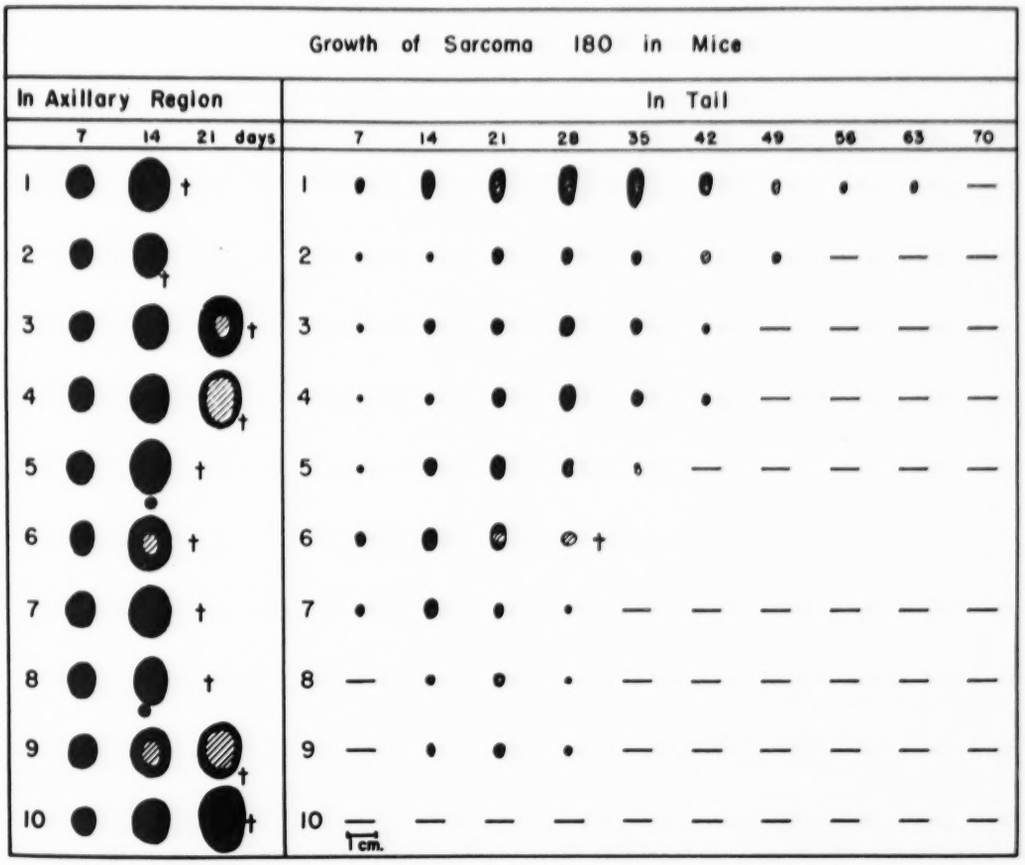


FIG. 1.—Area diagrams showing the effects of tail-pressure on the growth of Sarcoma 180

high-pressure steel pipe, 6 in. in diameter and 14 in. long. The caps and flanges were triple welded. This chamber was used for pressures up to 360 pounds per square inch. (A similar, smaller chamber, without a light or window, was used for high pressure work, before the one illustrated in Figs. 4 and 5 was available.) The flange of the high pressure chamber in Figure 4 was made of $1\frac{1}{4}$-in. steel, and its weight made it necessary to mount it on hinges. A lucite window was fitted. The insulated wire leading to the electric light was led in between two layers of a rubber gasket. The chamber end of the outlet tube was screened with wire, after several occasions when an inquisitive mouse was impacted into it. The inlet tube required no screening and was a simple length of small-diameter copper tubing similar to that used in compressed-gas manifold tubing, welded in. The outlet tube led to a Y fitting with a gauge on one arm and an adjustable needle-valve for the exhaust on the other. The pressure was controlled and maintained by the commercial reducing valve apparatus illustrated in Figure 5. Other features are illustrated in the photograph.

Effect of pressure on transplantability of Sarcoma 180.—In six experiments Sarcoma 180 was compressed just before transplantation to determine the effect of compression on transplantability. All mice used were of the Rockland Farms albino strain. In each case, two mice were selected in which the tumors were growing well in the usual axillary site 7 days after implantation. Both mice were killed simultaneously. One was subjected to compression for a varying period of time, and then a small piece (weighing about 6 mg.) of each tumor was implanted, by the usual trocar technic, into ten mice. Where hemorrhage was noted in the post-compression tumors, a nonhemorrhagic area was used for transplantation. The control mouse was left outside, on the top of the chamber. When

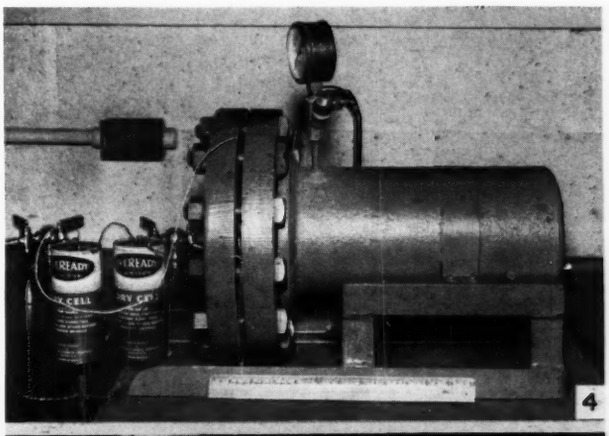
FIG. 2.—Sarcoma 180—16 days after implantation in the tail. The tumor shows marked hemorrhage.

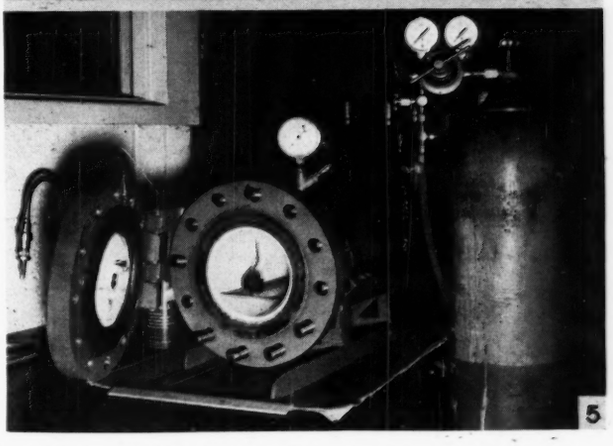
FIG. 3.—Same mouse 41 days later. There is complete absorption of the tumor and absence of ulcer. The animal is of normal appearance.

the high compression chamber was used, the control mouse was left in the low pressure chamber with the flange open. Both mice were killed at the same time, and both tumors were transplanted at the same time. One or more out of each ten mice transplanted with compressed tumor failed to show tumor growth; all the controls grew (Table 2). Both the compressed tumors and the controls were somewhat smaller than normal, as measured at 1 week, particularly if the mouse had been dead for 6 hours, or more, before transplantation.[1]

Effect of increased atmospheric pressure on growth of Sarcoma 180.—The effect of increased atmospheric pressure on the growth of Sarcoma 180, implanted into the usual axillary site, is summarized in Table 3. Pressures of 30 pounds per square inch were obtained from the building's piped compressed air supply. All other pressures were obtained by using air, or other gases, as supplied in the usual 220-cubic-foot compressed gas tanks. All gas mixtures used in this experiment were supplied ready-mixed in the tanks by the manufacturer. The rate of flow was adjusted to exceed 4 cc. of air/gram of mouse/minute, or the equivalent in oxygen.

The mice in Experiment 2 were left in the chamber uninterruptedly for 7 days, with food and water supplied *ad libitum*. In all other experiments, except the controls, the mice were slowly decompressed every 2 days. The time of decompression from 30 pounds was 1 hour. Two hours were used to decompress the mice from gauge pressures of 140 or 185 pounds; $2\frac{1}{2}$ hours from 318 pounds. As decompression proceeded below 50 or 70 pounds the *tum* were placed in the chamber, and the mice were immediately recompressed, the process taking 5 or 10 minutes. As the pressure increased, the mice would scratch at their ears but seemed otherwise unaffected. At high pressures (beginning at 300 pounds, most marked at 360 pounds), the mice would exhibit ataxia and a staggering gait and would rub at their noses.





FIGS. 4 and 5.—Photographs of compression chamber

TABLE 2

THE EFFECT OF INCREASED ATMOSPHERIC PRESSURE ON TRANSPLANTABILITY OF SARCOMA 180

Exp. no.	Gauge (pressure-pounds)	Time (hours)	Decompression (minutes)	Gas	Tumor takes / Tumor transplants	Tumor takes / Tumor transplants (Controls)
1	80	5	50	Air	8/10	10/10
2	105	4	65	Air	5/10	10/10
3	110	4	45	Air	9/10	10/10
4	195–110	$4\frac{2}{3}$	30	Air	8/10	10/10
5	210	$6\frac{3}{4}$	15	Air	6/10	10/10
6	285	$6\frac{1}{4}$	10	33 per cent air 66 per cent nitrogen	9/10	10/10
				Totals:	75 per cent	100 per cent

gas used was changed to compressed air. The chamber was then cleaned, a supply of Purina laboratory chow (2 gm./mouse/day) and water *ad libi-*

The 2 gm. of food/mouse/day were completely eaten. In earlier experiments it was found that, when more food was given, it was not always fin-

[1] The duration of viability of the tumor in the dead host was previously established for mouse sarcoma 180 (10). The transplantability of mouse sarcoma 180 was not changed when the tumor remained in the host for 9 hours at 22° C. after the host had been killed by a blow or by ether anesthesia.

ished. Loss of weight was a constant finding in all the mice in Table 3, averaging 20–25 per cent. Even if food was supplied *ad libitum*, weight loss under pressure occurred in all experiments, described in this paper, which lasted more than 1–2 hours.

pheric pressures of 140 pounds/sq. inch and over inhibit the growth of Sarcoma 180. The growth of these tumors is shown graphically in Figure 6. All tumors resumed growth at the normal rate when returned to normal pressure, with the possible exception of those exposed to 318 pounds (Fig. 6).

TABLE 3

EFFECT OF INCREASED ATMOSPHERIC PRESSURE ON GROWTH OF SARCOMA 180

Exp. no.	Number of tumors	Gauge (pounds)	Gas	Partial pressure of oxygen (lb.)	Time under pressure (days)	Food (gm/mouse/day)	Effect at end of 1 week
1	5	0	66 per cent oxygen 33 per cent nitrogen	10	7	2	No inhibition
2	6	30	Air	9	7	*ad libitum*	No inhibition
3	5	140	33 per cent air 66 per cent nitrogen	10	7	2	Moderate inhibition
3A	same 5 as in No. 3	30	Air	9	7	2	Resumed growth at normal rate
4	5	185	25 per cent air 75 per cent nitrogen	10	7	2	Slight inhibition
5	5	260–18	15 per cent air 85 per cent nitrogen	10	7	2	Marked inhibition

All the experiments summarized in Table 3 were done under the same partial pressure of oxygen but differ in total pressure. The first experiment confirms the findings of Campbell and Cramer that increase in the partial pressure of oxygen, by itself, has little effect on tumor growth.

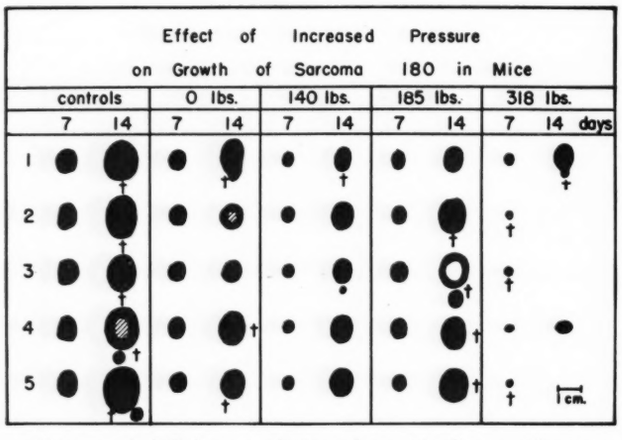


FIG. 6.—A definite retardation of growth of mouse Sarcoma 180 in mice subjected to atmospheric pressures of 140 pounds and over is shown. Shaded areas indicate hemorrhage or scab formation. 0 pounds: 66 per cent oxygen plus 33 per cent nitrogen; 140 pounds: 33 per cent air plus 66 per cent nitrogen; 185 pounds: 25 per cent air plus 75 per cent nitrogen; 318 pounds: 15 per cent air plus 85 per cent nitrogen.

No inhibition of growth was found after 1 week at 30 pounds gauge pressure of compressed air. In this experiment compression was started the day after transplantation of the tumor. In all other experiments in Table 3 the compression was begun the day of transplantation.

Experiments 3, 4, and 5 indicated that atmos-

After 1 week at 140 pounds per square inch, the mice in Experiment 3 were placed in compressed air at 30 pounds /sq. inch for 1 week, as indicated in Experiment 3A, and these mice, too, resumed apparently normal growth under 30 pounds pressure.

The mice in Experiment 5 were compressed to 360 pounds for the first 24 hours, then to 318 pounds for the remaining 6 days. At these pressures, 1 week of exposure proved quite toxic. One of the mice died after 5 days, and two more died at the end of the treatment. No hemorrhage was visible in any of the tumors in Table 3 at the completion of treatment, with the single exception indicated under 318 pounds in Figure 6, which showed partial hemorrhage. None of these tumors regressed.

Effect of increased atmospheric pressure on hemorrhage in Sarcoma 180.—In the earlier portion of this part of the work, dilution of air with nitrogen was obtained by connecting one tank of compressed air with one or more of nitrogen, by means of manifold tubing, with the resulting mixture fed to the compression chamber. This procedure was discontinued, subsequently, the tanks being ready-mixed by the manufacturer. Experiments 1 and 2 in Table 4 summarize all the experiments done with manifold mixed gases at pressures and durations within the indicated limits. Experiment 1 represents the aggregate of ten separate experiments. All others in this table represent experiments done with gases ready-mixed in the tank by the manufacturer. In all experiments in Table 4, except the anoxic ones, the rate of flow of the gas

was adjusted to exceed 4 cc. of air/gram of mouse/minute, or its equivalent in oxygen. This optimum flow was not always maintained (particularly in Exp. 1) due to gradual clogging of the outlet valve. All mice were implanted with a single tumor in the right axillary region.

The great majority of the mice in Experiment 1 died of oxygen poisoning. When the windowless chamber, which was being used at that time, was opened, they were found dead, with distended abdomens, rigidly outstretched hind legs, and coagulated froth exuded from the nostrils. The lungs showed marked congestion, and, as indicated in the table, most tumors showed marked hemorrhage. Marked hemorrhage (HHH) was considered a hemorrhage which converted all or most of the tumor into a grossly hemorrhagic mass (Fig. 7). It was usually visible through the skin, if the tumor were of sufficient size, and frequently the blood seemed under tension and would spurt out as the tumor was divided by the knife. Mild hemorrhage (HH) was considered a hemorrhage which involved less than $\frac{1}{2}$ the tumor. The symbol (H) in Table 4 represents a hemorrhage around the

TABLE 4

HEMORRHAGE IN SARCOMA 180

Exp. no.	Number of tumors	Age of tumors (days)	Gauge pressure (lb.)	Gas mixture (ratio of air/nitrogen)	Partial pressure oxygen (lb.)	Time at pressure (hours)	Time of decompression (minutes)	Hemorrhage*	Remarks
1	60	2–8	285–360	33/66 20/80	15–23	11–18	90–110	HHH	Aggregate of ten experiments. H–HHH in all. Mostly HHH; most dead at end of experiment.
2	8	9	300	helium air 80/20	12	16	70	HHH	All dead at end. $\frac{5}{8}$ HHH.
3	5	2	6	oxygen	21	$15\frac{3}{4}$	0	0	$\frac{1}{5}$ dead at end. No hemorrhage in any.
4	5	4	310	15/85	10	$\frac{1}{2}$	2	H	Killed by decompression. All had hemorrhage around tumor.
5	5	4	310	15/85	10	$\frac{1}{2}$	140	H	Killed with ether. Four had hemorrhage around tumor.
6	5	5	360	15/85	10.8	2	12	H	Killed by decompression. Four had hemorrhage around tumor.
7	5	7	315	15/85	10	5	15	0	Two killed by decompression; three killed with ether. No hemorrhage.
8	6	8	318	15/85	10	6.5	13	0	Three killed by decompression; three killed with ether. No hemorrhage.
9	5	5	60	oxygen	75	2	20	0	Died under pressure. No hemorrhage.
10	5	5	60	oxygen	75	$1\frac{3}{4}$	0	0	Died under pressure. No hemorrhage.
11	5	7	60	oxygen	75	$1\frac{1}{3}$	0	0	Died under pressure. No hemorrhage.
12	10	7	315	50/50	33	$2\frac{3}{4}$	45	HHH	Died under pressure. All marked hemorrhage.
13	5	5	345	50/50	36	$1\frac{3}{4}$	10	HHH	Two died under pressure; two mice HHH, 3 mice H.
14	5	5	320	50/50	33	$3\frac{1}{2}$	5	HHH	Two died under pressure; two mice HHH, one H.
15	5	7	315	50/50	33	$3\frac{1}{4}$	10	HHH	One died under pressure. Four killed by decompression, two mice HHH, one H.
16	5	4	18	oxygen	33	$3\frac{1}{2}$	0	0	Killed with ether. No hemorrhage.
17	5	7	18	oxygen	33	$3\frac{1}{4}$	10	0	Killed with ether. No hemorrhage.
18	5	7	30–45	oxygen	45–60	5	0	HHH	Four died under pressure; one died shortly. All HHH.
19	5	7	40	oxygen	55	$3\frac{1}{3}$	0	HHH	All died under pressure. All HHH.
20	5	7	315	15/85 & nitrogen	below 10	$5\frac{3}{4}$	13	HHH	Anoxic death under pressure. All HHH.
21	7	7	40	"	below 1.7	3	10	HHH	Two anoxic death under pressure after 3 hours. HHH. Five died after 1 hour and left in chamber 2 hours more—no hemorrhage.
22	4	7	300	"	low	3	10	HH	Anoxic death under pressure. Mild hemorrhage.
23	5	7	200	"	low	$5\frac{2}{3}$	10	HH	Anoxic death under pressure. Mild hemorrhage.
24	5	7	0	50/50 and 15/85	low	$\frac{1}{2}$	0	0	Four anoxic death without pressure. No hemorrhage.
25	6	7	0	"	low	5	0	0	Anoxic death without pressure. No hemorrhage.

*H: hemorrhage around tumor.
HH: mild hemorrhage.
HHH: marked hemorrhage.

tumor, in the tumor bed, and not in the tumor itself. Apparently, the hemorrhage produced in these experiments always began at the periphery of the tumor. Central hemorrhage, if noted, was considered spontaneous in all cases. In some experiments, some of the mice showed marked hemorrhage, and some showed little, or merely a hemorrhage in the tumor bed. These differences were sometimes thought due to differences in time of death (as in Exp. 21) but more often were thought to be due to individual variations (compare Exps. 12 and 15).

Histological examinations of a number of tumors in animals subjected to high pressures (Figs. 8 and 9) showed an abnormal vascularity, with dilated capillaries filled with blood. There were areas where the capillary walls had broken down and blood had entered the tumor tissue. Erythrocytes and sarcoma cells were here freely and diffusely mixed. At the edge of the Sarcoma 180, near the subcutaneous tissue, there were small areas of tumor necrosis but most of each tumor was viable and actively growing.

Nearly all the mice summarized in Experiment 1 were killed by their exposure, apparently from oxygen poisoning. Exposure to the same conditions, including the same partial pressure of oxygen, but with lower total pressures, caused neither death nor tumor hemorrhage (Exp. 3). When helium was substituted for nitrogen as a diluent, no difference was noted (Exp. 2). Apparently an increase in total pressure increases the toxicity of oxygen or is itself toxic.

Experiments 4–25 were carried out under observation in the windowed chamber illustrated in Figure 3. In Experiments 4–8 the tumorous mice were exposed to pressures of 300–360 pounds/sq. inch for $\frac{1}{2}$–$6\frac{1}{2}$ hours. These conditions were not

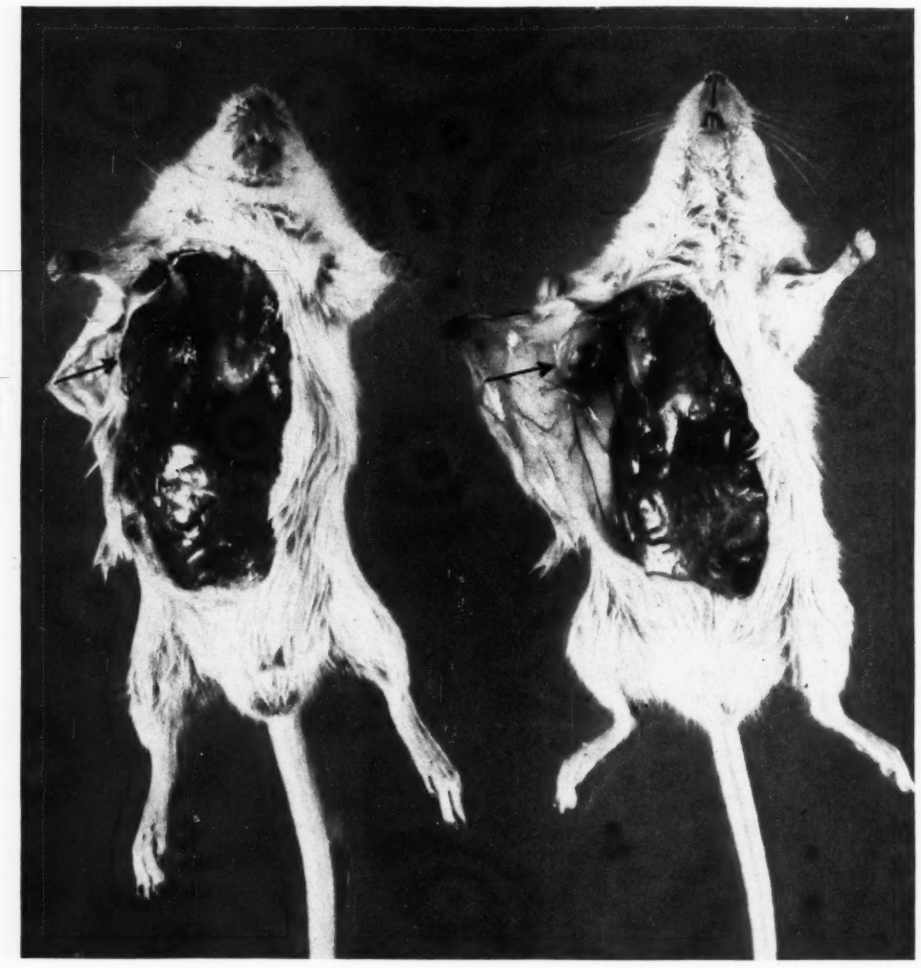


FIG. 7.—Photograph of the marked hemorrhage in Sarcoma 180 in a treated mouse (300 pounds pressure, 50 per cent air and 50 per cent nitrogen, $5\frac{2}{3}$ hours exposure) is contrasted with the absence of hemorrhage in the tumor of a control mouse (right side). Arrows point to the tumors.

lethal for the mice, some of which were killed by rapid decompression and some slowly decompressed and killed with ether. The time of decompression seemed to make no difference in the degree of hemorrhage, which appeared in about half of the experiments, and was restricted to the tumor-bed.

Experiments 9–11 summarize experimental conditions similar to those used by de Almeida (1) and Campbell (4). After some time, at 60 pounds gauge-pressure of oxygen, these mice would become extremely hyperactive, even frenzied, and would die in convulsions before 2 hours had elapsed. None of the tumors showed hemorrhage. These results are not comparable, since different tumors were used, but seem to resemble Campbell's, in the absence of tumor hemorrhage.

Experiments 12–15 again demonstrate the occurrence of marked tumor hemorrhage when death by oxygen poisoning takes place under high pressures. The mice in Experiments 10 and 13 were implanted with fragments of the same parent tumor, and this is also true in Experiments 14 and 16. Examination of these experiments indicates that tumor hemorrhage is related to the total pressure rather than the partial pressure of oxygen.

In Experiments 18 and 19 the animals were killed by oxygen poisoning in an atmosphere of pure oxygen. The pressure here was considerably lower than that used in Experiments 9–11; hence, the mice died more slowly. Marked tumor hemorrhage appeared in these mice.

The remaining experiments in Table 4 indicate that the production of tumor hemorrhage does not require oxygen poisoning but may accompany exposure to pressure under anoxic conditions. There is, again, some individual variation in response, due to factors which are not well understood, in the degree of hemorrhage produced under these conditions. (These individual differences also appeared in the tail implantations outlined in Table 1. They may be related to factors of immunity and resistance.)

The mice in Experiment 20 were brought to the indicated pressure using nitrogen with a 15 per cent admixture of air. After 2 hours the oxygen content of the chamber gas was reduced by gradual replacement with pure nitrogen, and this was continued until the animals died some 3 hours later. In the other anoxic experiments, the proportions of pure nitrogen, and of nitrogen with 15 per cent admixture of air, were adjusted to the point where

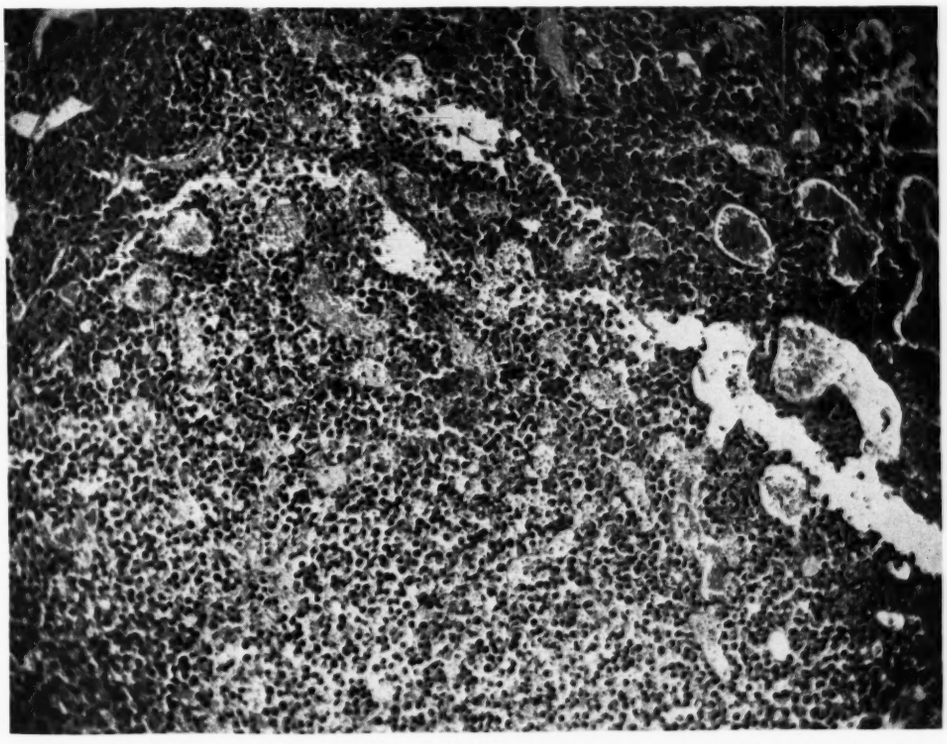


FIG. 8.—Histological appearance of treated Sarcoma 180 illustrated in Fig. 7. Sarcoma 180 shows an abnormal vascularization with dilated capillaries filled with blood. There are areas where the capillary wall has broken down and blood has entered the tumor. Hematoxylin and eosin stain. ×150.

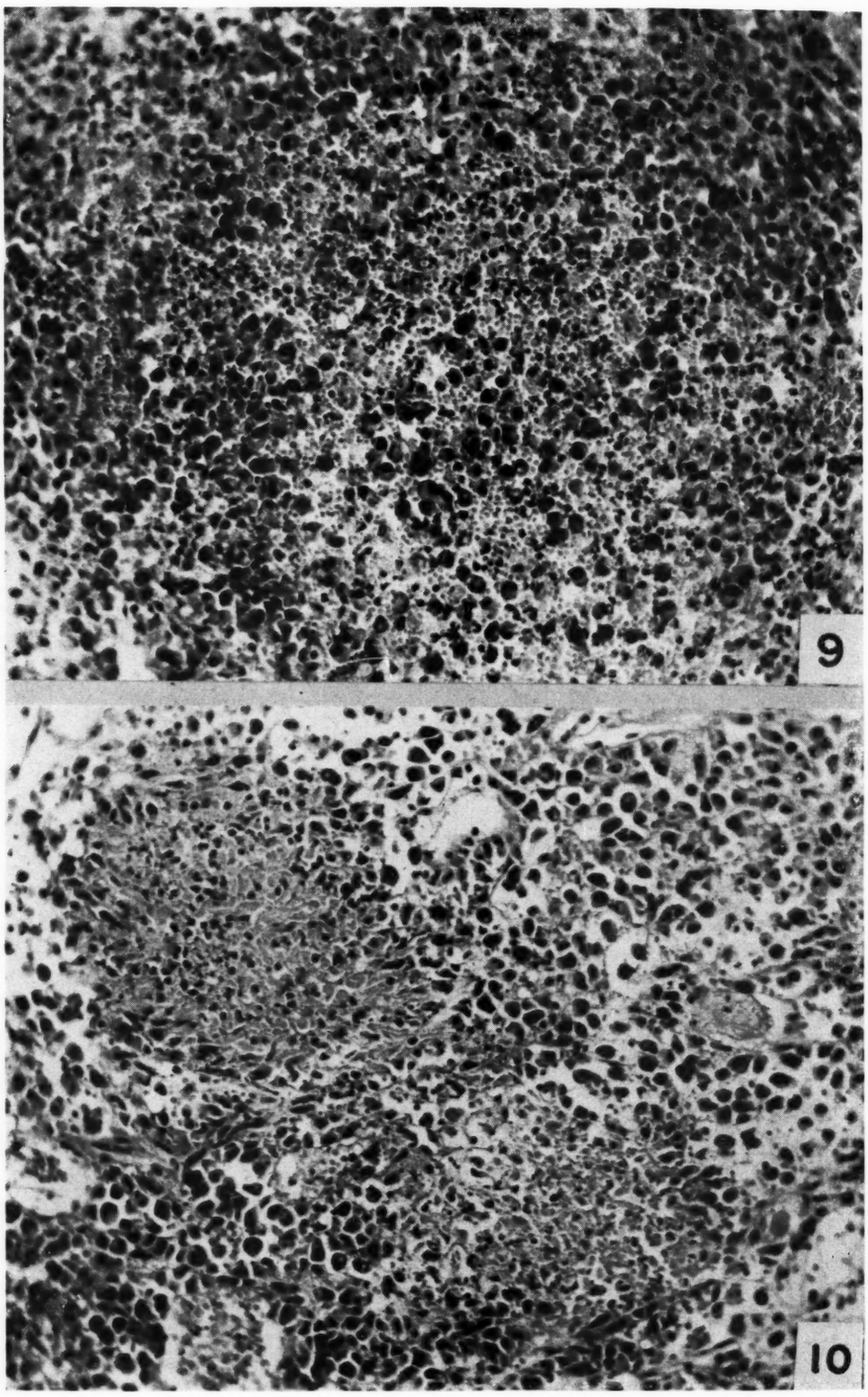


FIG. 9.—Higher-power view of Fig. 8 to show hemorrhage into the tumor. ×240.

FIG. 10.—Higher-power view of Fig. 8 to show necrosis in the tumor. ×240.

respiratory distress was evidence by slow, deep, gasping respirations, dizziness, and incoordination. The mice were maintained in this state by varying the proportions of the gases, and were asphyxiated, at the end of the interval indicated in the table, by introducing pure nitrogen. The mixtures were alternated by using a tank of each gas, both connected to the reducing valve by manifold tubing, and the valves of the tanks were opened alternately to furnish the desired gas. Control experiments at atmospheric pressure were done with an equal mixture of nitrogen and air. The control mice were asphyxiated by the 15 per cent air-nitrogen mixture (lethal at this pressure), at the end of the indicated time.

In these anoxic experiments the rate of air-flow fell below the level of 4 cc/gram of mouse/minutes of air, or the equivalent in oxygen. Hence, the expired CO_2 was not washed out of the chamber to the same extent as in the previous experiments. It is not known whether the increased CO_2 concentration played any part in the production of hemorrhage. However, in Experiment 23 hemorrhage appeared, despite the inclusion of "Baralime" in the chamber to absorb carbon dioxide.

In brief, the experiments detailed in Table 4 seem to indicate that tumor hemorrhage occurs when the experimental animal is killed, or almost killed, under the indicated conditions of pressure, either by oxygen poisoning or by anoxia, over a period of time exceeding approximately 3 hours. If one assumes that hemorrhage is an indication of some damage to the tumor (note the frequency of regression associated with hemorrhage both clinically and in Table 4), then conditions of increased pressure make the tumor more susceptible to damage by the two agents tested. However, with both anoxia and oxygen poisoning, the margin of safety under the conditions outlined was narrow, so that tumor hemorrhage was best obtained only when the organism itself was brought to the point of death (and, in some cases, not even then).

In all the experiments, the effect of pressure seems to have been concentrated on the tumor or the region immediately around the tumor. The other tissues and organs remained grossly and histologically unaffected by pressure of 300 pounds, except for loss of body weight, which was a constant finding. Congestion and hemorrhage of the lungs was noted whenever the oxygen concentration was high and was not related to the total pressure.

A small number of spontaneous mammary tumors in mice were exposed to pressures of 105 pounds/sq. inch, 50 per cent air and 50 per cent nitrogen, for 18 hours *in vivo*. Hemorrhage appeared during compression and was visible through the skin, upon removal from the chamber. All survived compression.

DISCUSSION

The experiments described in this paper confirm the clinical observation that pressure inhibits tumor growth. It is emphasized that this inhibition is due to the pressure itself and not to any variations in the concentrations of component gases. The following theory of pressure's mode of action is proposed as a working hypothesis, with the stipulation that many other factors, not discussed here, play major roles in growth and metabolism.

The effects of pressure on chemical reactions are described by Le Châtelier's Law, which states that the equilibrium of a system, when disturbed by a stress, is displaced in such a way as to tend to relieve the stress. Thus, pressure would affect any reaction in the growth process which is accompanied by volumetric change, inhibiting those which lead to an increase in volume. (The hydrolysis of certain proteins, for example, [6, 9] is accompanied by a decrease in volume.)

It is suggested that intracellular or intranuclear pressures (hence, the surface tension in their walls) may, through this mechanism, play a part in physiological as well as pathological metabolic processes of the cell contents.

SUMMARY

Sarcoma 180 was subjected to increased local compression, by implantation into the tail of mice of the Rockland Farms albino strain, with resulting retardation of tumor growth, increase in the frequency of tumor hemorrhage, and increase in the regression rate.

Compression decreased the transplantability of this tumor, under the conditions outlined.

Compression of the tumor-bearing mice, *in vivo*, resulted in retardation of tumor growth and increased susceptibility to tumor hemorrhage.

These effects seem to have been due to compression itself and not to any variation in the concentration of oxygen.

ACKNOWLEDGMENTS

It is a pleasure to acknowledge our indebtedness to Doctor C. P. Rhoads for his interest and encouragement.

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Effect of Partial Ablation of the Bone Marrow and of Splenectomy on the Hepatic Lesions Produced by 2-Acetaminofluorene*

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Bielschowsky (1) reported the occurrence of leukemia in rats receiving 2-acetaminofluorene in the diet. We have not encountered a single instance of this condition in over 2,000 rats (Wistar descendants and Sherman) treated with this agent under somewhat different experimental conditions (5, 7). Inasmuch as partial ablation of the accessible marrow has been reported to promote extramedullary myelopoiesis in the rabbit (6), 2-acetaminofluorene (AAF) was fed to rats subjected to this procedure in order to observe its effect upon extramedullary hematopoietic foci and the incidence of leukemia. In a number of rats, the partial ablation of the accessible marrow was preceded by splenectomy.

MATERIAL AND METHODS

One hundred and eighty-seven rats (Wistar descendants and Sherman) were divided into the following groups: *Group 1 (AAF–Albation)*: 23 males and 14 females (80–110 gm.) received 2-acetaminofluorene (AAF) incorporated in their food (2) in a concentration of 0.03 per cent for 120 days and, subsequently, the same diet without AAF. In all animals, large portions of the accessible marrow were destroyed and replaced by inert material, as indicated below. At 6–8-day intervals during the first 3 weeks of the experimental period, the femora, tibiae, and humeri were exposed, an opening was made with a dental drill, and the marrow was destroyed. High-frequency current was used for this purpose, at first, but was abandoned because of uncontrollable burns. The marrow was destroyed mechanically, and bleeding was well controlled by oxycel.[1] Thermosetting plastic[2] and radio-opaque glass-wool[3] were packed into the cavity. For effective packing in the humeri and tibiae, steel wire was also inserted, occasionally. This was especially helpful when fractures occurred. The tails of a number of male rats were amputated just above the anal opening, at the beginning of the experiment. A number of animals died from infection as a result of the operations and were discarded.

Group 2 (Ablation controls): 31 males and 7 females were subjected to the ablation procedure, as indicated. They received no AAF.

Group 3 (AAF controls): 86 male and 10 female rats received AAF, but no marrow ablations were performed.

Group 4 (AAF-Ablation-splenectomy): The spleens were removed from 11 male rats, and the animals were then subjected to the ablation procedure and given the carcinogen.

Group 5 (Ablation-Splenectomy Controls): 5 male rats were subjected to splenectomy and marrow ablation and were given no AAF.

These 16 rats subjected to splenectomy remained free from Bartonella infection, whereas others, treated by the same procedure, were discarded because of supervening Bartonella infection.

Hematological studies were made at regular intervals, with tail blood and, at the time of sacrifice, with heart blood. Hemoglobin determinations, and erythrocyte, leukocyte, and differential counts were performed, with heparin as an anticoagulant.

The animals were sacrificed after 29–360 days, and tissues from the parenchymatous organs were fixed in Bouin's solution and neutral formolalcohol and stained with hematoxylin and eosin.

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[1] Parke Davis and Co., Detroit, Mich.

[2] Styrene monomer, The Dow Chemical Co., Midland, Mich.

[3] Owens-Corning, Fiberglass Co., Toledo, Ohio.

RESULTS

Hematological observations.—The animals exhibited a permanent leukocytosis following partial ablation of the marrow. The degree of leukocytosis fluctuated widely. In one AAF-ablation male, there was leukopenia. The leukocytosis was associated with myeloid immaturity. The values for hemoglobin and red blood cells also fluctuated widely, and several animals showed a severe degree of anemia. The changes in females were less marked than in males. All animals subjected to ablation showed these changes, whether or not AAF was fed.

Extramedullary myelopoiesis.—Histological examination revealed a constant occurrence of extramedullary myelopoietic foci in the spleen, while these foci occurred less frequently and to a lesser degree in the liver and kidneys. These extramedullary foci were characterized by groups of myeloid cells, normoblasts, and megakaryocytes. There was no difference, in this regard, between the animals of group 1 (ablation-AAF) and group 2 (ablation-controls).

Spleen.—No consistent changes were observed in the size of the spleen. The extent of extramedullary myelopoiesis varied considerably, but in no instance was this marked enough to obscure the lymphoid architecture of the spleen. The myeloid foci contained promyelocytes, myelocytes, and megakaryocytes, either in small groups or dispersed throughout the splenic pulp.

Liver.—In the rats subjected to partial bone-marrow ablation (with or without splenectomy) and subsequently fed AAF, liver tumors developed much earlier than in the control (non-ablated) groups receiving AAF. As indicated in Table 1, the incidence of malignant hepatoma after 160 days of treatment with the carcinogen was 30 per cent in the control groups (no ablation) and 100 per cent in animals treated for an equal length of time but deprived of a large proportion of their bone marrow. The data on liver weight, given in the same table, indicate that these tumors not only appeared earlier but also grew more rapidly. When treatment with AAF is extended to 350 days, almost all male rats develop liver tumors (5), whether or not they were subjected to bone-marrow ablation. However, it would appear that even in these late stages the growth of the liver tumor is greater in the "ablated" animals receiving the carcinogen than in controls (no ablation), as evidenced by comparison of the liver weights. Partial ablation of the bone marrow, alone, without administration of the carcinogen, did not cause enlargement of the liver (Figs. 1–4).

Proliferation of hepatic reticulum cells and intrahepatic bile ducts was a constant and striking early finding in the AAF-treated, marrow-ablation group. The earliest changes in this group consisted of extensive proliferation of pale, oval or elongated cells with indistinct cytoplasm and palely staining nuclei, some resembling reticuloendothelial cells and others bile-duct cells (Fig. 5). Following parenteral administration, trypan blue and thorotrast were taken up by many of these cells (Fig. 8).

TABLE 1

INCIDENCE OF HEPATOMA AND THE SIZE OF LIVER IN MALE RATS

EXPERIMENTAL GROUPS	NUMBER OF ANIMALS	DURATION OF EXPERIMENT (days)	INCIDENCE OF HEPATIC TUMORS (per cent)	AVERAGE LIVER WEIGHT AS PER CENT OF BODY WEIGHT			
				Non-tumor	Range	Tumor	Range
Ablation+AAF	12	76–160	100			9.8	5.7–12.7
AAF	30	"	30	6.7	3.8–9.6	8.1	5.3–10.3
Ablation+AAF	7	160–350	100			29.4	20.7–35.9
AAF	56	"	91	6.3	3.6–9.0	12.8	5.4–28.0

Subsequent changes included bile-duct proliferation and increase in cellular connective tissue in the portal areas. The histologic picture resembled an early cirrhotic process. The liver cells showed varying degrees of regeneration, and hepatoma nodules were present at as early as 76 days in two instances (Fig. 7). The early proliferation of reticuloendothelium and bile ducts considerably exceeded the minor changes of similar nature observed in the animals of group 3 (AAF controls). The malignant hepatomas and cysts were similar to those previously described in AAF-treated rats (5, 2). These hepatic changes were accelerated still more when ablation of the accessible marrow was combined with splenectomy in AAF-fed rats (Figs. 6–8) Hepatoma was observed as early as 49 days, and cirrhosis was more marked than in the other group (Table 2). In female rats, however, ablation of the bone marrow did not intensify or otherwise modify the carcinogenic effect of AAF on the liver.

DISCUSSION

Steinberg (6) found that partial ablation of the bone marrow of rabbits was followed by the appearance of extramedullary myelopoietic foci. This

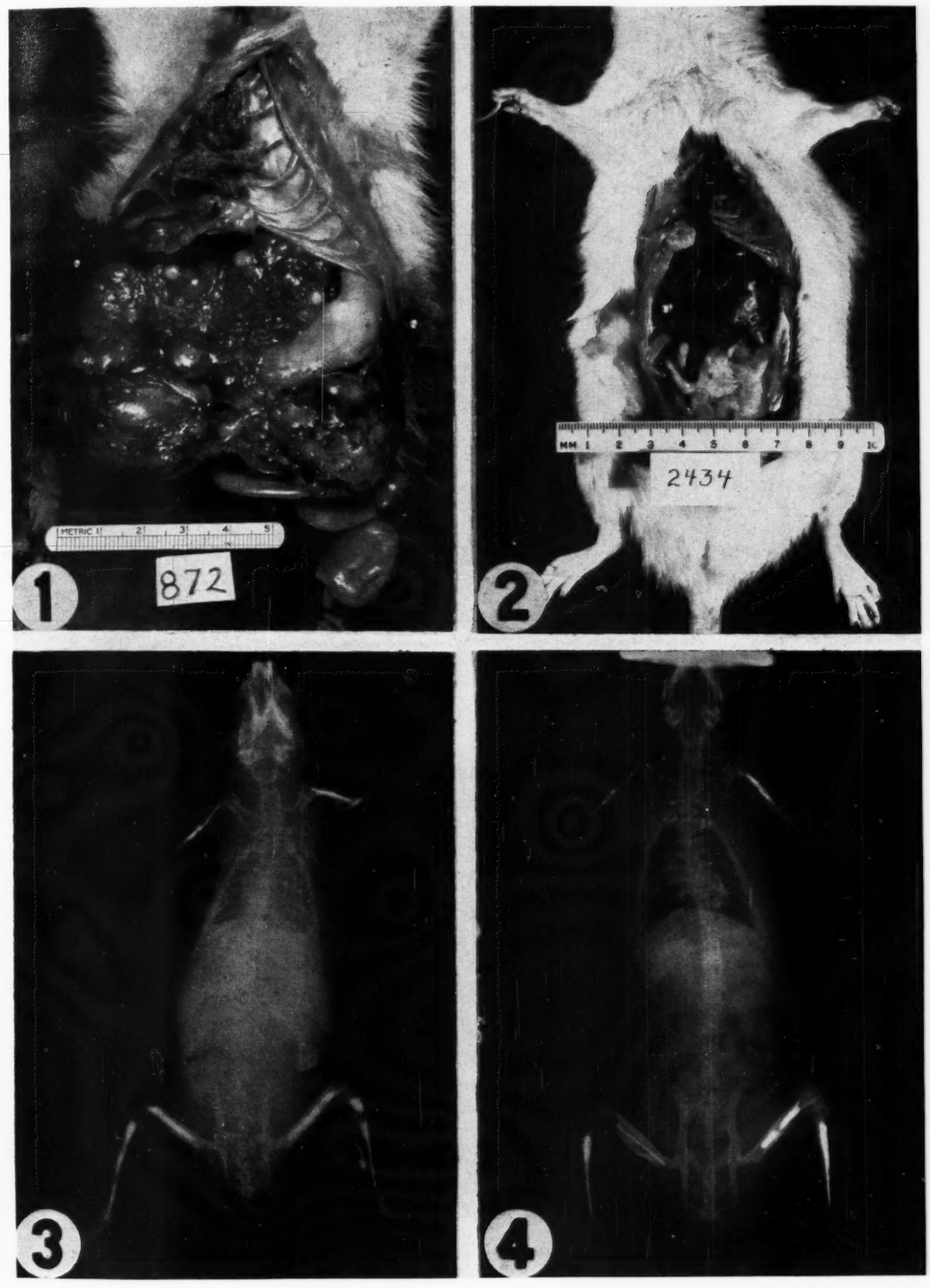
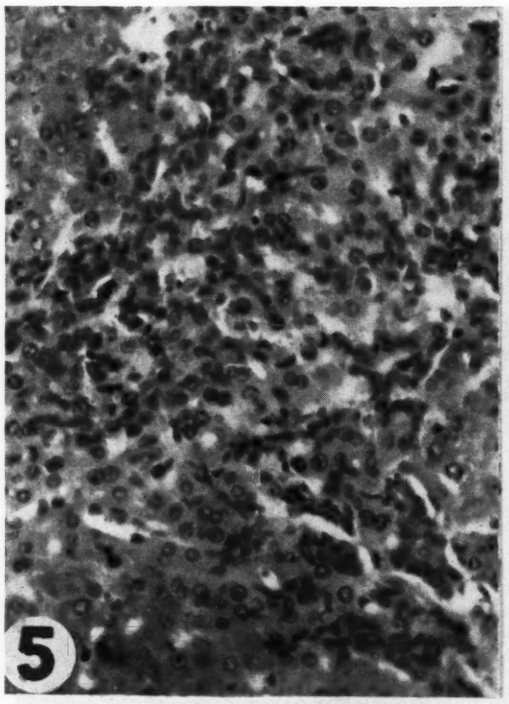


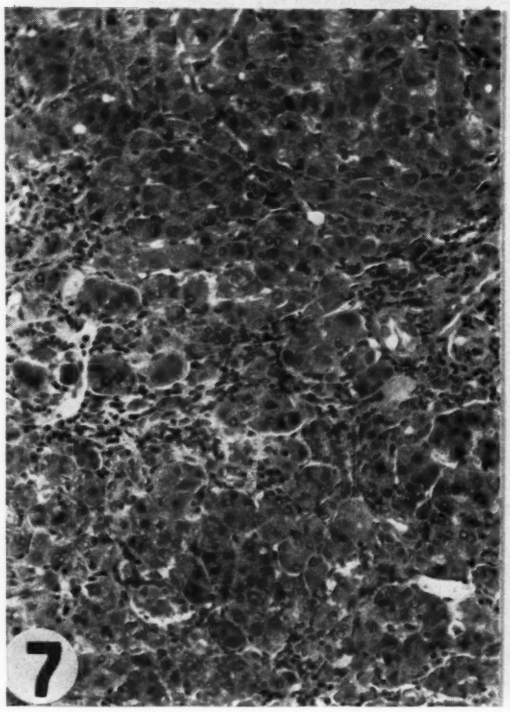
FIG. 1.—Rat 872. Bone marrow ablation, AAF for 215 days; compare size of livers and extensiveness of malignancy with that of rat 2434 (Fig. 2).

FIG. 2.—Rat 2434. No ablation. AAF for 215 days; compare with Fig. 1.

FIG. 3.—Rat 1211. Bone marrow ablation. Marrow cavity filled with radio-opaque material. AAF for 158 days. Roentgen photograph following thorotrast injection. The opaque material in the marrow cavity demonstrates the extent of marrow loss. Enlarged liver demonstrable due to thorotrast storage. Compare with Fig. 4.

FIG. 4.—Rat 1218. Bone marrow ablation. Marrow cavity filled with radio-opaque material. No carcinogen. Roentgen photograph after injection of thorotrast. Normal size of liver. Compare with Fig. 3.





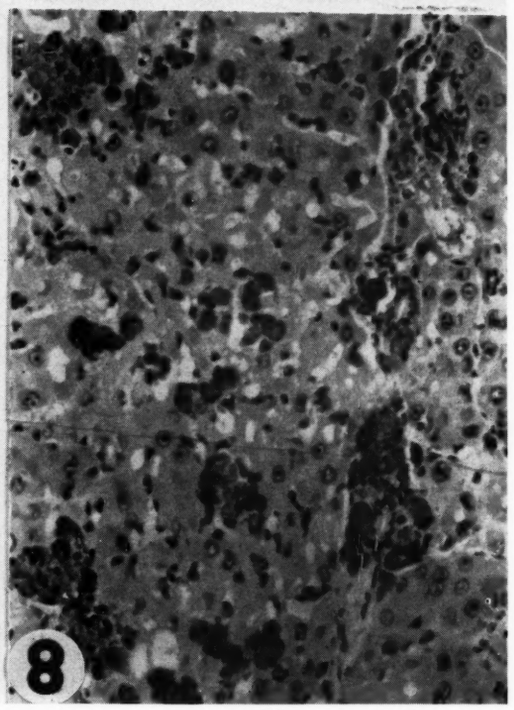


FIG. 5.—Rat 877. Bone marrow ablation. AAF for 29 days. Note early extensive proliferation of reticuloendothelial cells and bile-duct epithelium. 230 × Hemat.-Eosin.

FIG. 6.—Rat 1385. Bone marrow ablation and splenectomy. AAF for 105 days. Roentgen photograph following thorotrast injection. The opaque material in the marrow cavities demonstrates the extent of marrow loss. The liver is enlarged and nodular.

FIG. 7.—Rat 1385. AAF for 105 days. Bone marrow ablation and splenectomy. Note increase of young connective tissue, and the early development of malignant hepatoma. 125 × Hemat.-Eosin.

FIG. 8.—Rat 1382. AAF for 105 days. Bone marrow ablation and splenectomy. Injected with thorotrast 24 hours prior to sacrificing. Note pigment deposits, probably thorotrast, indicating the reticuloendothelial nature of the cells. 210 × Hemat.-Eosin.

author estimated that 41 ± 5 per cent of the total marrow was removed by destroying the marrow of both tibiae, femora, humeri, radii, and ulnae, the other bones being inaccessible with the method employed. Application of this ablation technic in the rat is more difficult, and we were able to apply it only to the femora, tibiae, and humeri. In a few groups of young animals, the tails were amputated to increase the amount of marrow removed. The studies of Dietz (3) indicate that 47 per cent of the marrow is located in the hind legs and 13 per cent in the forelegs. The marrow of the long bones is as active in the rat as in the rabbit (4). It seems logical to assume that the ablation technic employed in this study removed about 30–40 per cent of the marrow.

Neither ablation of the bone marrow nor ablation combined with splenectomy caused development of leukemia, as judged by the peripheral blood picture and by tissue sections. However, ablation of active bone marrow was followed, as expected, by evidences of increased regeneration. Leukemia has been reported in AAF-treated rats (1). However, this appears to be rare, and in more than 2,000 rats (Wistar and Sherman strains) treated with AAF in our laboratory, not a single instance of leukemia has been observed.

It was believed that the increased regeneration following bone-marrow ablation might possibly lead to leukemia in animals exposed to a carcinogen with as broad a range of action as AAF. This, however, was not the case. Similarly, in experiments reported previously (2), increased tissue growth induced in target organs by various hormones failed to become malignant in the presence of AAF.

From these observations, the conclusion appears justified that intensification of (normal) cell proliferation does not predispose a tissue to the development of cancer, even in the presence of a carcinogen which induces malignant growth elsewhere at the same time.

Reduction in the volume of active bone marrow, and without splenectomy, intensified the carcinogenic effect of AAF upon the liver, causing an earlier appearance and more extensive growth of cancer of that organ. It is noteworthy that this effect was observed only in male, and not in female, rats—the latter remaining more resistant to the carcinogen. This sex difference in incidence of liver cancer has been observed previously in rats receiving AAF (2).

SUMMARY

Large portions (about 30–40 per cent) of the accessible bone marrow of rats were destroyed and replaced by inert material; in some experiments, this procedure was combined with splenectomy. One group of animals received AAF in its diet. Control groups included AAF-treated intact rats and rats not receiving the carcinogen but subjected to bone-marrow ablation with and without splenectomy.

After ablation, these animals exhibited leukocytosis and slight extramedullary hematopoiesis, but no leukemia. Exposure of this vigorously regenerating hematopoietic system to AAF failed to induce leukemia. The significance of these findings in regard to the relation of intensified "normal" cell proliferation to carcinogenesis is discussed briefly.

Cancer of the liver, induced by feeding AAF, appeared earlier and grew more extensively in male rats subjected to bone-marrow ablation, or to the latter combined with splenectomy, than in intact animals receiving the carcinogen. Female rats, whose livers are less susceptible to the carcinogenic action of AAF, failed to exhibit intensification of liver cancer under the conditions of the experiment.

TABLE 2

HEPATIC CHANGES INDUCED IN MALE RATS BY AAF AND PARTIAL BONE MARROW ABLATION AND SPLENECTOMY

Experiment	Number of animals	Duration of experiment (days)	Proliferation of reticulo-endothelium bile duct	Early "cirrhosis"	Hepatoma
AAF	34	34–106	9	13	0
Ablation+AAF	7	29–106	7	5	3
Ablation+AAF+splenectomy	9	21–106	9	9	6
Ablation	8	22–106	1	0	0
Ablation+splenectomy	5	23–106	0	0	0

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Histological Changes Produced by a Single Large Injection of Radioactive Phosphorus (P^{32}) in Albino Rats and in C3H Mice*

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Numerous reports have appeared, describing the therapeutic use of radioactive phosphorus (P^{32}) in polycythemia vera (5–7, 10, 11, 15, 19, 22, 23), leukemia (4, 5, 7, 9, 13–16, 19, 22–25), and other conditions (10, 12, 22). However, there have been few systematic investigations of the effects of radiophosphorus on the histological appearance of organs and tissues (1, 8, 21). An attempt was therefore made to discover the immediate effects of P^{32} on cytological structures and the location of the effects throughout the body by means of histological studies.

METHODS

Twenty-two normal albino rats and eight C3H mice bearing mammary tumors were injected subcutaneously with a single large dose of P^{32} administered as phosphoric acid without carrier. The dose administered to each animal, the time after injection when the animal was sacrificed, and other details are described in Table 1. The animals were killed with chloroform, and tissues were fixed in Heidenhain's Susa and stained with hematoxylin-eosin for histological examination. Attempts were also made to localize the administered P^{32} in the tissues by means of the Geiger counter and the autographic technic, but these results will be published separately.

RESULTS

In general, the effects of P^{32} were most evident in causing damage and destruction in the nuclei of cells. The nuclear changes consisted chiefly of pyknosis and karyorrhexis of mitotic nuclei. Metabolic nuclei appeared to enlarge, to become pale and vesicular, and to contain prominent eosinophilic nucleoli. In the cytoplasm, changes in the ability to take up stain and vacuolation were seen in damaged and recovering cells, and some loss of polarity of cell constituents; e.g., in the position of the Golgi apparatus in the intestinal epithelium.

Little change was observed in those structures which might be grouped as structural elements. Thus, no effect was noted on the fibrous elements of connective tissue, nor on the cellular elements (fibroblasts, mast cells, macrophages, etc.). The myofibrils of muscle also showed no change. The effects on bone were not examined in this investigation, but Bloom noted some dead cartilage cells in the epiphyseal plate of a P^{32}-injected mouse (2). Little change was observed in cartilaginous tissue of our animals, either in the chondrocytes or in the perichondrium. The epidermis showed no evidence of damage, but pyknosis and nuclear dust were visible in the cells of the hair root follicle as early as 1 day after injection and as late as 9 days.

The cerebrum and the cerebellum showed no changes, and nervous tissue in general appeared very resistant to P^{32}.

A slight inflammatory reaction in the lungs was seen 12 and 24 hours after injection, as indicated by a sticking of neutrophils and other white blood cells to the endothelium of small venules in the lung tissue, but with little infiltration of the lung parenchyma. No changes in the bronchioles or trachea were noted in our animals.

No changes were seen in the kidneys of these animals, in any parts of the nephron, or blood vessels, or in the bladder.

Among the tissues which synthesize secretions, the exocrine glands were very resistant. Thus, no radiation damage was visible in the salivary glands of the animals examined, in the lacrimals, or in the accessory male sex organs.

The only change that may be said to have occurred in the pancreas due to the administration of P^{32} is the appearance of pyknotic acinar cells which were widely scattered throughout the tissue. However, in the rat this reaction is not specific for

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P^{32}, but occurs in response to other types of trauma as well.

No alterations were noted in the histological structure of the liver except in one mouse 10 days after injection of the radioactive phosphorus. Necrosis of the cells around the central veins was visible, and the sinusoids were abnormally wide. The damaged cells had indistinct outlines—a bright eosinophilic homogeneous cytoplasm and pyknotic and karyorrhectic nuclei.

TABLE 1

ADMINISTRATION OF P^{32} TO ALBINO RATS* AND TUMOR-BEARING C3H MICE

Millicuries of P^{32}	No. of animals	Sex	Time interval between injection and sacrifice
1.0	2	F, M	10 minutes
2.0	1	F	" "
1.25	1	M	2 hours
1.4	1	M	" "
2.0	1	F	" "
2.4	1	F	" "
4.0	1	M	" "
1.0	1	M	12 "
2.0, 2.0†	1	M	19 "
1.25	1	M	24 "
1.6	1	M	" "
2.0	3	F, F; M	" "
2.4	1	F	" "
3.0	1	F	" "
4.0	1	M	" "
less than 1.25	1	M	48 "
1.25	1	M	" "
4.0	1	M	3 days
1.0	1	F	8 days
		C3H Mice‡	
0.0	1	F	Control
1.0	1	F	2 hours
0.51	2	F	24 hours
1.8	1	F	" "
1.0	1	F	48 "
1.3	1	F	7 days
1.8	1	F	9 days

* All rats weighed 50–60 gm. except those injected with 4.0 mc.—which weighed 140 gm.

† 60-gm. rat which received two doses of P^{32}—one of 2.0 mc. at 0 hours, and another of 2.0 mc. at 11 hours—and was sacrificed at 19 hours.

‡ All mice were adult and weighed 30–40 gm.

In the endocrine glands, no changes were observed in the pituitary, thyroid, or adrenal glands. However, in the gonads, although no changes occurred in cells in the metabolic state, functioning as hormone producers, some radiation effects were observed in the cells undergoing mitotic and meiotic divisions. Thus, in the testis, the Leydig and Sertoli cells remained unaffected. Only the germinal cells were damaged and these not until 8 days after P^{32} injection. In one rat, the changes involved a few spermatogonia and some primary spermatocytes. In some instances, these spermatocytes showed very abnormal meiotic figures and also nuclear irregularities in the resting forms such as binucleatism, with two separate nuclei, or two nuclei joined by strands, giant cells, and other freakish structures. Some spermatocytes also were dead and pyknotic. In other cases, there was some chromatolysis of nuclei of spermatocytes. There were only minimal changes in the rest of the male genital system.

The first unequivocal changes in the histology of the ovary appeared 24 hours after P^{32} administration. In the graafian follicles, numerous cells throughout the granulosa were pyknotic and degenerating; large droplets of nuclear and cytoplasmic debris floated free in the liquor folliculi. In follicles where the destruction of the granulosa was almost complete, the whole follicle had a slightly collapsed and irregular outline, the basement membrane becoming indistinct in places. Some of the smaller follicles showed similar changes in the granulosa; others just beginning to undergo involution and atresia showed none. Primary follicles, newly formed corpora lutea, and interstitial cells remained unaffected at 24 hours. However, at 48 hours some very small follicles showed some pyknotic changes. No changes were found in the germinal epithelium or in the uteri.

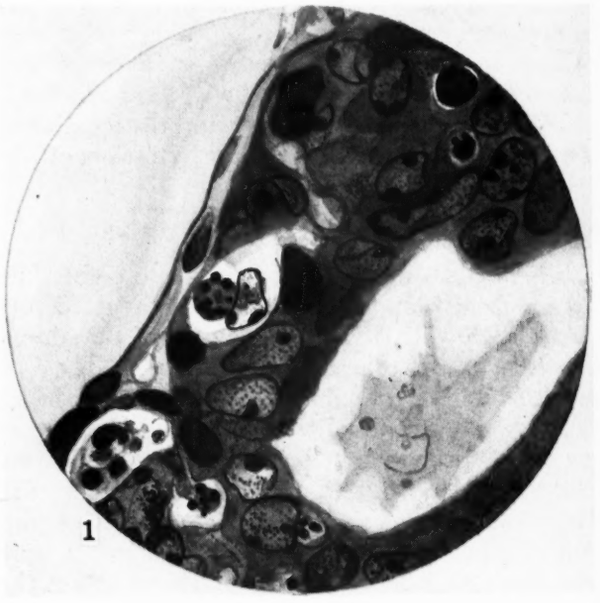


FIG. 1.—High magnification drawing of a portion of a spontaneous mammary tumor in a C3H mouse which received 1.0 mc. of P^{32} before sacrifice. Several cells show various types of cytological changes and pyknotic degeneration due to the effect of P^{32}.

The injection of P^{32} had no damaging effect on the normal mammary tissue of C3H mice bearing mammary tumors; however, the effect of this radioactive isotope of phosphorus on the spon-

FIG. 2.—Section of the thymus of a rat which received 2.0 mc. of P^{32}, 2 hours before sacrifice. Low magnification. At this magnification the thymus is normal in appearance. The dark staining cortex is packed with lymphocytes, the lobules are large. Microscopically, scattered pyknotic lymphocytes are found in the cortex.

FIG. 3.—Section of the thymus of a rat which received 2.0 mc. of P^{32}, 12 hours before sacrifice. Low magnification. Signs of P^{32} damage are seen in this thymus. The size of the individual lobules, the thickness of the cortex, and its staining intensity are much reduced because of a marked reduction in the number of lymphocytes present. Microscopically, almost all the lymphocytes are seen to be pyknotic.

FIG. 4.—Section of the thymus of a rat which received 2.0 mc. of P^{32}, 48 hours before sacrifice. Low magnification. Radiation damage has resulted in a loss of all distinction between cortex and medulla. The dark-stained areas represent groups of completely pyknotic lymphocytes, and the remainder, the reticular supporting tissue of the thymus and some resorbed fat tissue.

FIG. 5.—Section of the thymus of a larger rat which received 4.0 mc. of P^{32}, 3 days before sacrifice. Low magnification. Practically no lymphocytes remain in the thymus; only a small amount of reticular and reduced fat tissue is found.

taneously developing tumor itself was striking. The first changes were observed at 24 hours, when numerous pyknoses were present instead of the mitoses seen in the tumors of the control animals (Fig. 1). There also appeared to be a greater amount of necrosis than in the controls. At 48 hours, there was an increase in the number of blood lakes, both large and small, and some clotting of blood occurred. Still later, there was an increase in degeneration and liquefaction. At 7 and 9 days, some of the tumors were almost totally necrotic and liquefied. No direct effect of P^{32} was visible on the individual endothelial cells.

The histological changes observed in the hemopoietic organs were striking. The earliest changes observed in the bone marrow were seen 18–48 hours after injection of the isotope and consisted of a great thinning of the myeloid elements. Among the remaining elements, mature red blood cells and megakaryocytes predominated, but the immature white blood cells disappeared at a slightly slower rate than the immature red blood cells. By 3 days after injection, the bone marrow consisted mainly of sinuses filled with mature red blood cells and a very small amount of connective tissue stroma containing monocytes and macrophages and the occasional megakaryocyte. The changes in the lymph nodes were first seen 24 hours after P^{32} administration, at which time there was a great loss of lymphocytes from the entire gland, and particularly from the nodules, while many of the remaining lymphocytes showed pyknosis or karyorrhexis. From the second to the ninth day, the nodular structure was entirely absent, but masses of macrophages and monocytes were seen between the large, pale reticular cells. In the thymus (Figs. 2–5) the destruction of lymphocytes was even more evident, and the effects were observed earlier (at the 12-hour interval). Some loss of lymphocytes in the malpighian corpuscles of the spleen was also noted 12 hours after the injection of P^{32}. The changes seen in the subsequent intervals are essentially the same as those seen in the other lymphatic organs.

In the gastro-intestinal tract, the effect of P^{32} on the eosophagus was negligible. In the stomach, mitoses normally occurring at the bases of the gastric pits among the mucous neck cells were replaced by pyknotic cells 24 hours after the injection of P^{32}. However, the other cell types of the epithelium were not affected. Small areas of hemorrhage were observed in the juxta-pyloric region.

The most striking changes seen in the gastro-intestinal tract were noted in the epithelium of the small intestine (Figs. 6–13). Two hours after P^{32} injection, the only change visible was found in the crypts, where an occasional cell with a pyknotic nucleus could be seen among the numerous mitoses which occur normally. By 12–24 hours, the crypts were full of pyknotic nuclei, and the very few mitoses which were apparent showed abnormalities (Fig. 8). The total number of cells in the crypts was reduced. The Paneth cells remained normal in appearance at this time interval, but the goblet cells were much enlarged, pale, and vesicular, with several large eosinophilic nucleoli. These abnormal cells occurred throughout the crypts, and a few were also found forming the epithelium at the base of the villi. Above these, the villus cells were normal, except that the goblet cells were much distended with mucus.

At 48 hours there were already some signs of recovery, since a few normal mitoses started to appear in the middle portion of the crypts (Fig. 9). Pyknoses, however, were still very numerous, especially in the lower part of the crypts, but the number of such damaged cells was reduced. Enlarged cells with unusually pale nuclei and large nucleoli could be seen in the crypts and as high as halfway up the villi. The Golgi apparatus of these cells did not appear in the normal position which is immediately apical to the nucleus.

At 72 hours, the thickness of the mucosa was definitely greater. However, the picture seen at this time was far from normal. The total number of cells in the crypts was much decreased; there was often a wide lumen, and the epithelium was thin and wavy in outline. The cells varied in height from the normal columnar to low cuboidal—the total size, however, being much increased. The nuclei were often large and pale, and many showed three or four very large nucleoli. Some giant cell forms and binucleate cells were present, and the goblet cells were very numerous. The epithelium of the villi was also very much thinned and the total number of cells reduced (Figs. 6 and 7). In the ileum the enlarged goblet cells caused a marked bulging, since the epithelium between them was low cuboidal in form, with very large, pale, round nuclei (Fig. 13). Goblet cells were not present in the duodenum, but marked degenerative changes were seen in the other cells of the villi. The cells were irregularly shaped and the nuclei often shrunken, especially toward the tips of the villi. These villus cells had a finely vacuolated cytoplasm, which was also most obvious toward the tips of the villi (Fig. 11). However, in spite of the very abnormal picture 3 days after the administration of P^{32}, the picture of the whole mucosa after 8 days had returned completely to normal.

Other changes occasionally noted in the small intestine were edema and engorgement of the ves-

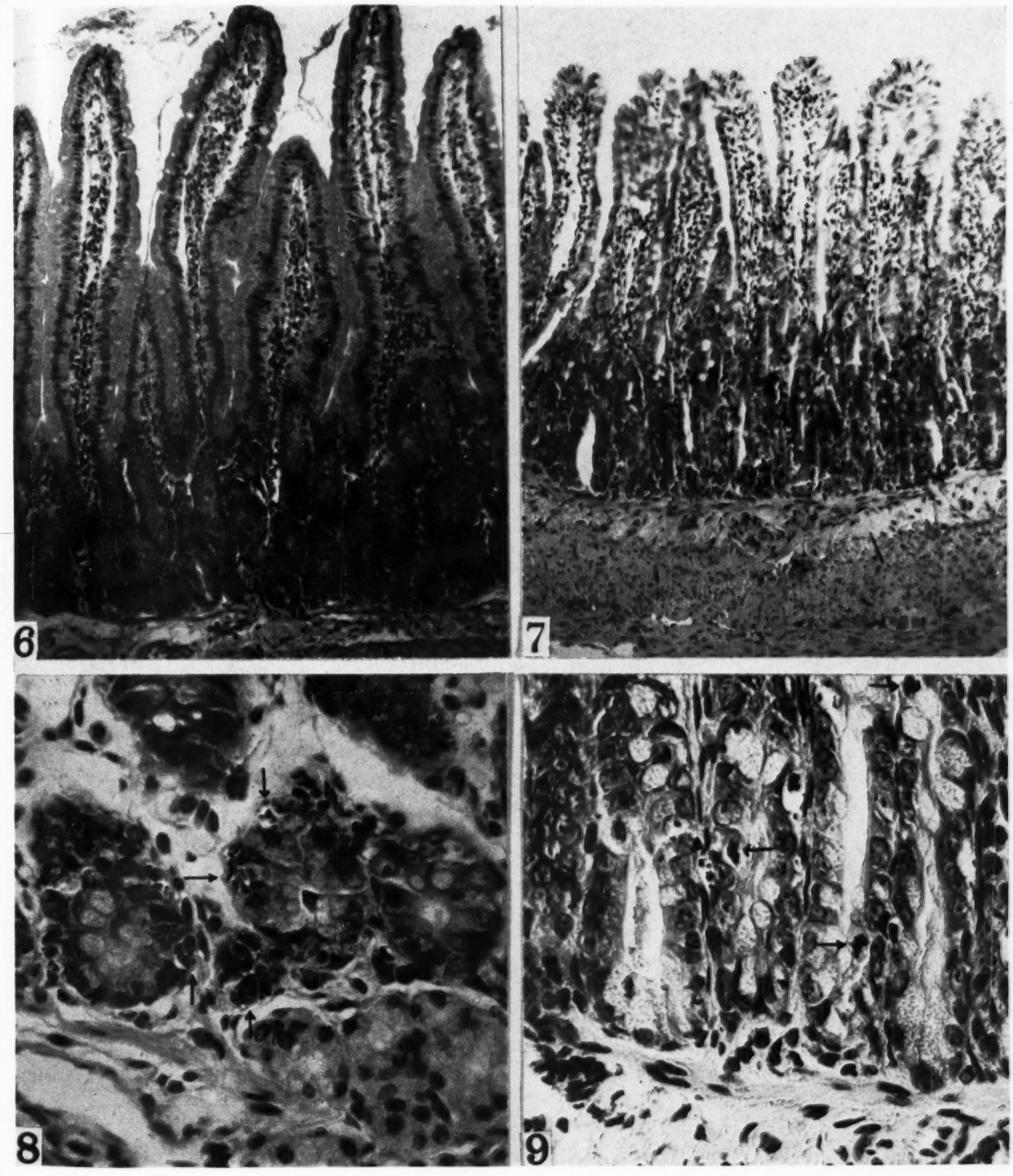
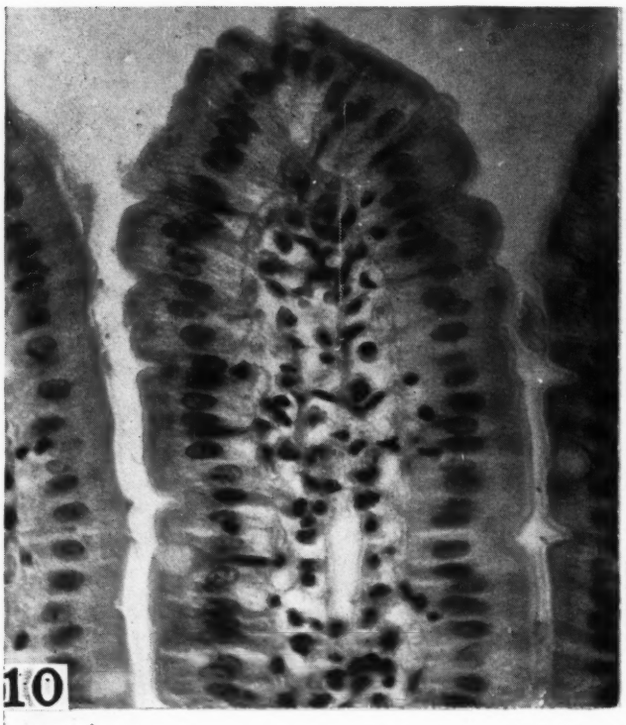


FIG. 6.—Section of the duodenum of a rat which received 1.0 mc. of P^{32}, 5 minutes before sacrifice. Low magnification. In this normal duodenum, note the height of the villi and crypts, also the thickness and regularity of the epithelial cells.

FIG. 7.—Section of the duodenum of a rat which received 4.0 mc. of P^{32}, 3 days before sacrifice. Low magnification. The damaging effects of the P^{32} radiation are shown by the great decrease in the over-all thickness of the mucosa. The epithelial cells are very much reduced in height, and their nuclei are widely and irregularly spaced.

FIG. 8.—Section of the duodenum of a rat which received 4.0 mc. of P^{32}, 24 hours before sacrifice. High magnification. Several cross sections of the bases of the crypts of Lieberkuhn show a complete absence of mitotic figures, which are replaced by a large number of pyknotic cells, indicated by arrows. The Brunner's glands below the crypts appear completely normal; they do not normally show mitoses.

FIG. 9.—Section of the duodenum of a rat which received 1.25 mc. of P^{32}, 48 hours before sacrifice. High magnification. Recovery from P^{32} damage in the crypts is shown by the presence of several normal mitoses, indicated by the arrows. However, some pyknotic cells are also seen, and there is a thinning of the whole epithelium with a relative increase in the number of goblet cells.



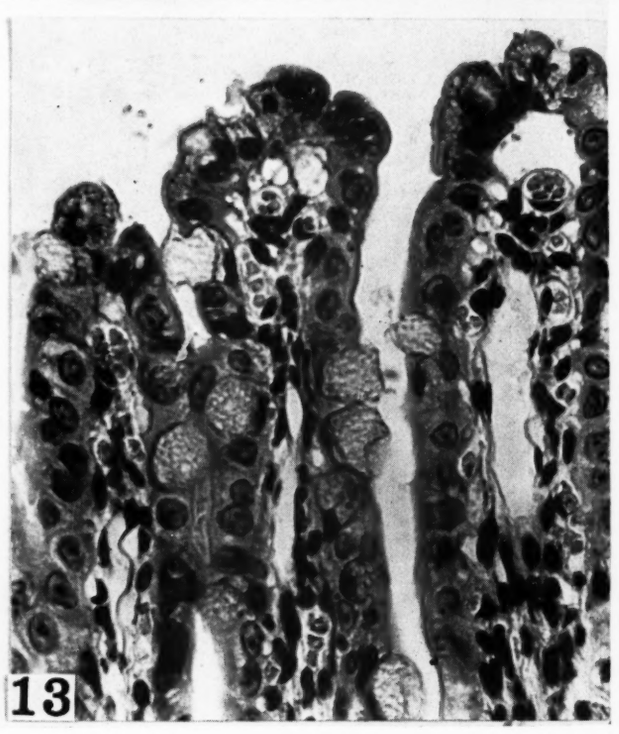


FIG. 10.—Section of a villus tip from the duodenum of a rat which received 2.0 m.c. of P^{32}, 5 minutes before sacrifice. High magnification. The epithelium of the villus has retained its normal appearance. The cells are tall columnar in form, closely packed and the nuclei evenly placed.

FIG. 11.—Section of a villus tip from the duodenum of a rat which received 4.0 mc. of P^{32}, 3 days before sacrifice. High magnification. The damaging effect of the P^{32} is shown by the extreme thinning and irregularity of the epithelium. The cells show very variable shapes, dark shrunken irregular nuclei and a fine vacuolization throughout their cytoplasm (shown clearly at the tip of the central villus). The striated border is present and unchanged, but follows the very irregular outline of the epithelium.

FIG. 12.—Section of the villus tip from the ileum of a rat which received 2.0 mc. of P^{32}, 2 hours before sacrifice. High magnification. The epithelium of the villus has retained its normal appearance. The cells are tall columnar in form, closely packed and the nuclei fairly evenly placed.

FIG. 13.—Section of the villus tip from the ileum of a rat which received 4.0 mc. of P^{32}, 3 days before sacrifice. High magnification. The damaging effect of the P^{32} is shown by the changes in the epithelium. The goblet cells appear tremendously swollen with mucus secretion. The chief cells are now very widely spaced and have a low cuboidal form, with rounded nuclei and a fine vacuolization of their cytoplasm.

sels of the submucosa and a reduction in the number of lymphocytes present in the villus. Macroscopically, some small hemorrhagic areas were observed. The Brunner's glands of the duodenum were not damaged.

In the colon there was pyknosis of a few cells in the crypts, and on recovery the number of goblet cells appeared to be increased.

DISCUSSION

The histological changes brought about by the administration of large doses of P^{32} to animals were very similar to those produced by intense treatment with x-rays (26). It has been shown previously that the effects of large doses of x-rays were either direct or indirect (17). The experiments with P^{32} reported here suggests that the changes observed in gastro-intestinal tract, mammary tumors, ovary, hair follicles, and bone marrow were due to a direct impact of the rays. This assumption is supported by unpublished results which show that these structures are among those with the highest concentration of P^{32} in the body. It is also known that these tissues have a high mitotic rate, which may render them particularly sensitive to ionizing radiations.

On the other hand, the lesions of the thymus and lymphatic system may be due to a nonspecific reaction such as is observed in many types of stress resulting in a release of adrenal hormone, which then acted on the thymicolymphatic system. Thus, Leblond and Segal (17) have demonstrated that thymic involution did not occur in intensively x-radiated adrenalectomized rats, if the thymus was shielded from direct destruction by the rays. However, such involution took place in intact animals, even if the thymus was protected. Examination of reports (20) of thymic involution due to massive x-radiation of hypophysectomized rats with hypofunctioning adrenals revealed that no precautions were taken in these experiments to shield the thymus against direct destruction by the rays. However, it is not unlikely that an additional direct effect of the rays of P^{32} may have contributed toward the destruction of the thymus, an organ with a high P^{32} content. The occasional hemorrhagic areas noted in the stomach might also be due to an indirect effect.

The changes observed in the villi of the small intestine can be explained as follows: a rapid cell formation takes place in the crypts, from which new cells move up into the villus epithelium. These are later extruded into the intestinal lumen from the villus tip (18). P^{32}, by destroying dividing cells in the crypts, reduces the supply of cells to the villus, presumably without decreasing the cell loss from the tip. As a result, a decrease in the total cell number would occur with a subsequent distortion of villus architecture (compare Figures 6, 10, and 12, with Figures 7, 11, and 13).

The dose of P^{32} may be of some importance in determining the type of histological change obtained. Thus, Bloom (1) administered P^{32} to mice in smaller doses than those reported here and did not observe any damage in the thymico-lymphatic system, Peyer's patches, or ovary. However, in his animals there was damage in the duodenum, bone marrow, and testis. The Paneth cells of the ileum also increased in number and size 15 days after the administration of the radioactive phosphorus (3). Platt, who used still smaller doses administered repeatedly to humans for many months, observed "fibrosis, hyalinization of collagen, and a vascular alteration characterized by thickening and hyalinization of small blood vessels" (21). However, in addition to the dose administered, species differences and the time in which P^{32} was allowed to act may also play a part in the different responses obtained.

CONCLUSIONS

Twenty-two normal young male and female albino rats and eight adult female C3H mice received a single large dose of radiophosphorus (P^{32}) and were sacrificed from 2 hours to 9 days later.

The cellular destructions consisted mostly of pyknosis and karyorrhexis of mitotic nuclei and enlargement of metabolic nuclei. The cytoplasm of damaged cells showed vacuolation and reduced staining ability.

The destructive effects of P^{32} were most marked in lymphatic organs and bone marrow, small intestine, granulosa of ovarian follicles, and mammary tumors.

In the crypts of the small intestine, massive degeneration and pyknosis of dividing cells resulted in thinning of the epithelium of crypts and villi, probably because no new cells were supplied from the crypts to balance the continual loss of cells by extrusion normally occurring at villi tips (18).

The mammary tumors showed many scattered pyknotic cells at 24 hours after injection. At 48 hours, large blood lakes had formed without much tissue damage. At 9 days, most of the tumor was necrotic and liquefied.

Lesser changes were noted in stomach and colon, in the seminiferous tubules of testis, and in the hair follicles.

Little or no damage was observed in salivary glands, pancreas, liver, genito-urinary system, endocrine glands (except the ovary), brain, muscular and connective tissue.

ACKNOWLEDGMENTS

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Neoplasms in Rats Treated with Pituitary Growth Hormone

I. Pulmonary and Lymphatic Tissues*

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The occurrence of certain neoplasms in rats injected for long periods of time with pituitary growth hormone has been previously reported (2). This finding was of such unusual interest that an additional group of rats was similarly treated for the purpose of studying neoplastic and hyperplastic changes in the tissues.

Fifteen female rats of the Long and Evans strain, 237–39 days old, whose weight had become stabilized, were injected intraperitoneally with pure pituitary growth hormone daily, 6 days weekly, for a maximum of 485 days. A similar group of adult females received, parenterally, comparable amounts of an inert protein. Eight of the growth hormone-injected rats were sacrificed 350–465 days after the beginning of the experiment because of their poor condition. Malignant neoplasms were found to be the cause of the poor health in six of the rats. Two rats in the control group were sacrificed at 378 and 438 days after the beginning of the experiment, and a malignant neoplasm was found in one of these. The remainder of the rats were sacrificed 483–85 days after the beginning of the experiment. At autopsy, the organs of all animals were examined with dissecting binocular microscopes. Representative portions of all organs were taken for microscopic study.

The increase in body weight and in the length of the rats injected with growth hormone (Fig. 1, Table 1) was similar to that observed in the rats previously reported (2). The rats in the present group received 0.4 mg. of growth hormone daily at the beginning of the experiment. The dosage of growth hormone was increased at intervals as indicated in Figure 1. In the latter part of the experiment, the rats were receiving 3 mg. daily. The control rats injected with albumin showed only a slight gain in weight, comparable to that seen in uninjected female rats. The response of the individual rats is given in Tables 1 and 2.

Although many neoplasms of various types occurred in the rats injected with growth hormone, this paper will deal only with the changes in the lungs and lymphatic tissues of these animals. The neoplastic and hyperplastic changes in other tissues will be reported subsequently.

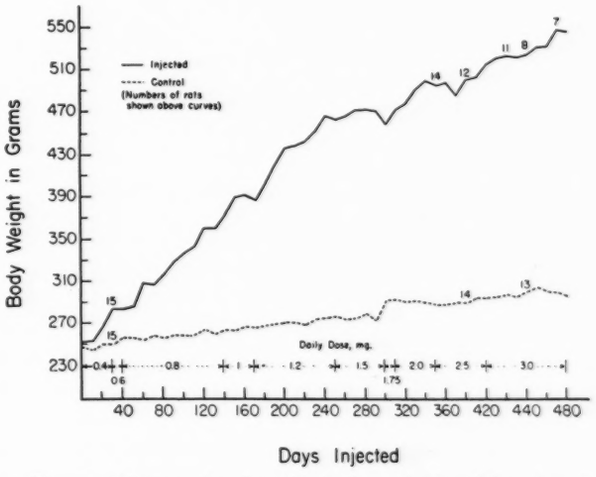

FIG. 1.—Curves showing the body weights of female rats chronically injected with pituitary growth hormone and their protein-injected controls.

RESULTS

RATS INJECTED WITH GROWTH HORMONE

Lungs.—All the rats injected with growth hormone showed an increased amount of peribronchial lymphoid tissue.

Lymphosarcomas, apparently arising in the lungs from peribronchial lymphoid tissue, were present in six of fifteen rats which were injected with growth hormone (Table 3). There was replacement of entire lobes by solid masses of soft

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pink tissue (Figs. 2–5 and 9–10). In one of these animals, B6458 (Fig. 2), there was erosion of the right main bronchus and obstruction of the lumen by soft neoplastic tissue. There was complete replacement of the right lung and partial replacement of the left lung in two rats. The involved portions of the lungs were greatly enlarged. The greatest involvement of pulmonary tissue occurred in the lower lobe of the right lung in five animals. In lungs showing incomplete replacement by lymphosarcoma, the tumor showed a peribronchial distribution (Figs. 14–16). The mediastinal and hilar lymph nodes were involved by tumor in four rats. In rat BH6274 (Figs. 6–8) the right pulmonary vein was invaded by tumor, and a small mass of the tumor extended into the left atrium.[1]

These pulmonary neoplasms consisted of cells varying from reticulo-endothelial cells to lymphocytes. The lymphoblast predominated in most tumors. These cells had a moderate amount of pale cytoplasm and large nuclei which were often hyperchromatic. Mitotic figures were numerous. Delicate strands of reticulum were present in a few areas, but there was no intimate relationship between the neoplastic cells and reticulum fibers.

The neoplastic reticulo-endothelial cells were

TABLE 1

BODY WEIGHT AND LENGTH OF FEMALE RATS CHRONICALLY INJECTED WITH PITUITARY GROWTH HORMONE
(Age at onset, 237–39 days)

ANIMAL NUMBER	AGE AT AUTOPSY (days)	DAYS INJECTED	BODY WEIGHT Onset (gm.)	BODY WEIGHT Autopsy (gm.)	BODY WEIGHT Gain (gm.)	LENGTH AT AUTOPSY (cm.)
G6534	587	350	236	430	194	43.4
B6458	615	378	256	391	135	42.3
GH6368	618	380	242	484	242	42.7
BH6274	651	412	235	469	234	45.5
BH6334	668	430	247	451	204	43.7
G6506	669	432	243	551	308	45.4
BH6276	673	435	269	506	237	43.4
G6553	702	465	235	425	190	42.8
BH6313	722	484	238	654	416	46.5
W6324	721	483	273	605	332	44.9
G6255	724	485	258	557	299	45.4
G6361	722	484	239	446	207	44.6
B6266	723	484	277	468	191	46.2
G6269	723	484	247	478	231	46.1
G6271	723	484	271	537	266	47.0
Av. 7:*			258	535	277	45.8

* Only data from rats examined at autopsy after 483–85 days of treatment are included in averages.

TABLE 2

BODY WEIGHT AND LENGTH OF NORMAL FEMALE CONTROLS
(Age at onset 237–39 days)

ANIMAL NUMBER	AGE AT AUTOPSY (days)	DAYS INJECTED	BODY WEIGHT Onset (gm.)	BODY WEIGHT Autopsy (gm.)	BODY WEIGHT Gain (gm.)	LENGTH AT AUTOPSY (cm.)
B6530	615	378	251	222	−29	32.1
B6512	672	435	239	260	21	39.8
B6400	722	484	239	264	25	40.6
(B6508)*	720	483	(277)	(441)	(174)	(40.0)
W6523	720	483	235	323	88	42.0
GH6442	722	485	236	267	31	40.3
GH6554	722	485	247	290	43	42.5
GH6357	722	484	260	341	81	42.3
G6460	722	485	257	336	79	41.5
(BH6262)*	722	483	(244)	(252)	(8)	(42.4)
G6268	723	484	242	281	39	40.9
B6291	722	484	234	289	55	42.0
BH6292	722	484	271	303	32	42.0
B6492	722	485	249	297	48	41.1
BH6294	723	485	235	272	37	40.7
Av. 11:			246	297	51	41.4

* With the exception of rats B6508 and BH6262, which had tumors of the pituitary, only the eleven rats examined at autopsy after 483–85 days of treatment are included in the averages.

[1] An attempt at transplantation of this tumor was unsuccessful.

considerably larger than the lymphoblasts. They had abundant azurophilic or basophilic cytoplasm. The nuclei were large and frequently irregular; the nuclear membranes were thick. A single prominent nucleolus was present in some of the nuclei. The chromatin was present in coarse clumps; mitoses were numerous. These cells occurred in compact clusters along a delicate reticulum framework. In some areas, neoplastic cells were surrounded by reticulum envelopes.

The neoplastic lymphocytes were larger than normal lymphocytes. They had very little cytoplasm and relatively large, hyperchromatic nuclei. Many of the nuclei were irregular. Occasional mitoses were present.

All tumors contained all three types of cells, representing various stages of differentiation. However, one cell type usually predominated and occurred in large masses. Interspersed throughout the tumor were small groups of neoplastic cells which were in different stages of differentiation. In some of the tumors, there were isolated macrophages which produced a "pock-marking" of the tissue. Most of these macrophages could be readily identified by their abundant cytoplasm which contained phagocytized particles. These macrophages were not considered neoplastic.

In two of the rats, focal areas of lymphosarcomatous transformation were noted in the lung opposite the one which contained the major lesion. Areas of such transformation occurred in the hyperplastic peribronchial lymphoid tissue. The lymphosarcomatous cells were easily distinguishable from the normal lymphocytes. The latter cells were displaced by the more rapidly proliferating neoplastic cells (Figs. 11–13).

Invasion of vascular channels by lymphosarcoma was seen in three rats; in one of these, the invasion was noted grossly. Groups of abnormal lymphatic cells similar to those seen in the pulmonary tumor occurred in the adrenal glands in one of these rats (BH6274). In no other instance were lymphosarcomatous cells observed outside the thoracic cage.

While many of the rats showed slight dilatation of the bronchi, and in two there was bronchopneumonia, there were no pulmonary lesions in any of them comparable to the bronchiectasis and squamous metaplasia of rats described by Passey, Leese, and Knox (6).

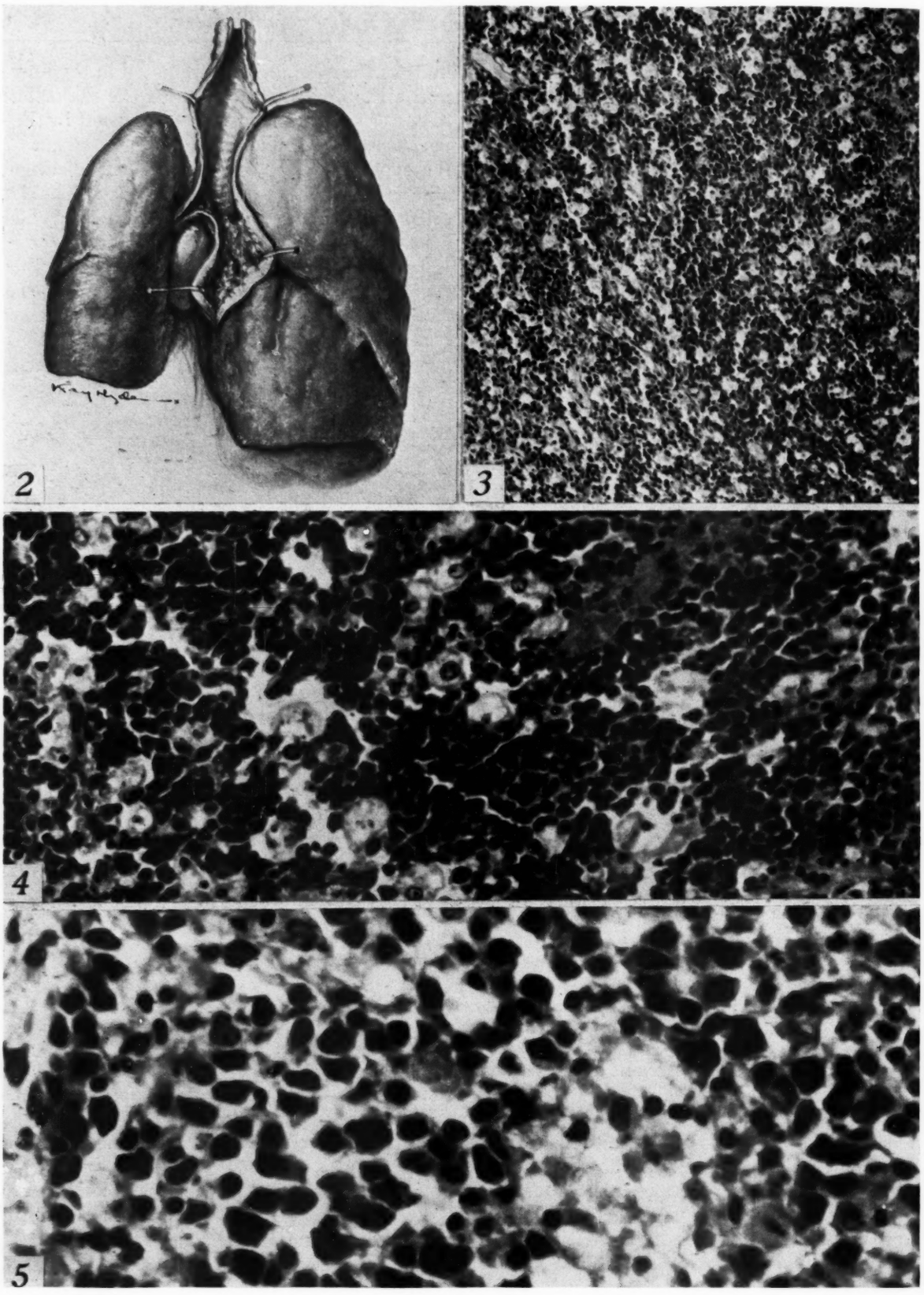
Lymphatic tissues.—As previously mentioned, the mediastinal and hilar lymph nodes of four of the rats with lymphosarcoma of the lungs were greatly enlarged (Figs. 2 and 6). Microscopically, they showed replacement by neoplastic lymphoid cells of the types seen in the pulmonary tumors.

TABLE 3

PULMONARY AND LYMPHATIC LESIONS OF RATS INJECTED WITH GROWTH HORMONE

Rats	Period of treatment (days)	Age at autopsy (days)	Lungs	Lymphatic tissues
G6534	350	587	peribronchial lymphoid hyperplasia	
B6458	378	615	lymphosarcoma	lymphosarcoma, hilar lymph nodes
GH6368	380	618	tumor of right lung*	
BH6274	412	641	lymphosarcoma	lymphosarcoma, hilar lymph nodes
BH6334	430	668	peribronchial lymphoid hyperplasia	
G6506	432	669	peribronchial lymphoid hyperplasia	
BH6276	435	673	peribronchial lymphoid hyperplasia	
G6553	465	702	lymphosarcoma	lymphosarcoma, hilar lymph nodes
BH6313	484	722	lymphosarcoma	
W6324	483	721	lymphosarcoma	lymphosarcoma, hilar lymph nodes
G6255	485	724	peribronchial lymphoid hyperplasia	
G6361	484	722	peribronchial lymphoid hyperplasia	
B6266	484	723	peribronchial lymphoid hyperplasia	
B6269	484	723	peribronchial lymphoid hyperplasia	
G6271	484	723	lymphosarcoma	

* The right lung of this animal was inadvertently discarded and was not examined histologically. For this reason the rat was not included with the six animals showing lymphosarcoma.



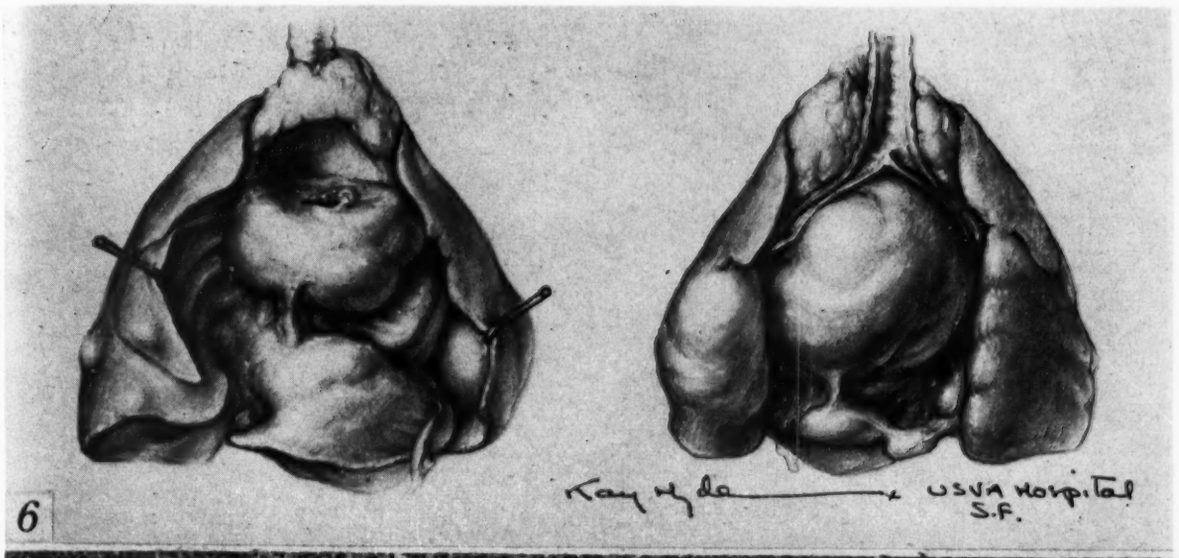
FIGS 2–5.—LUNG OF EXPERIMENTAL RAT B6458

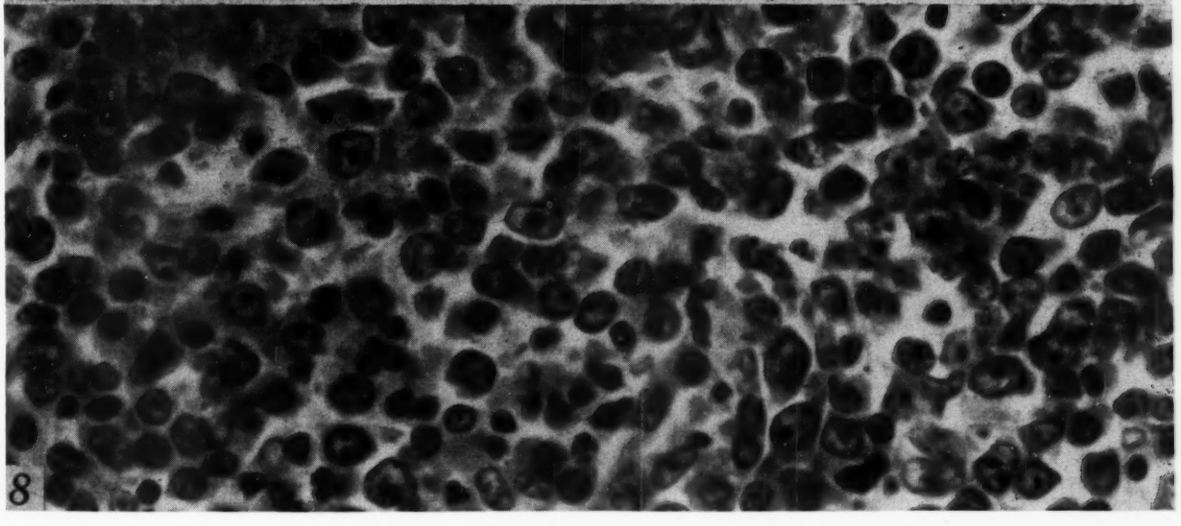
FIG. 2.—Artist's sketch of lungs; posterior view showing massive enlargement of right lung, erosion of right main bronchus and enlargement of hilar lymph node.

FIG. 3.—Photomicrograph of lymphosarcoma of right lung. ×125.

FIG. 4.—Note "pock-marking" of tumor by macrophages. ×355.

FIG. 5.—Note variation in size and shape of nuclei. ×800.



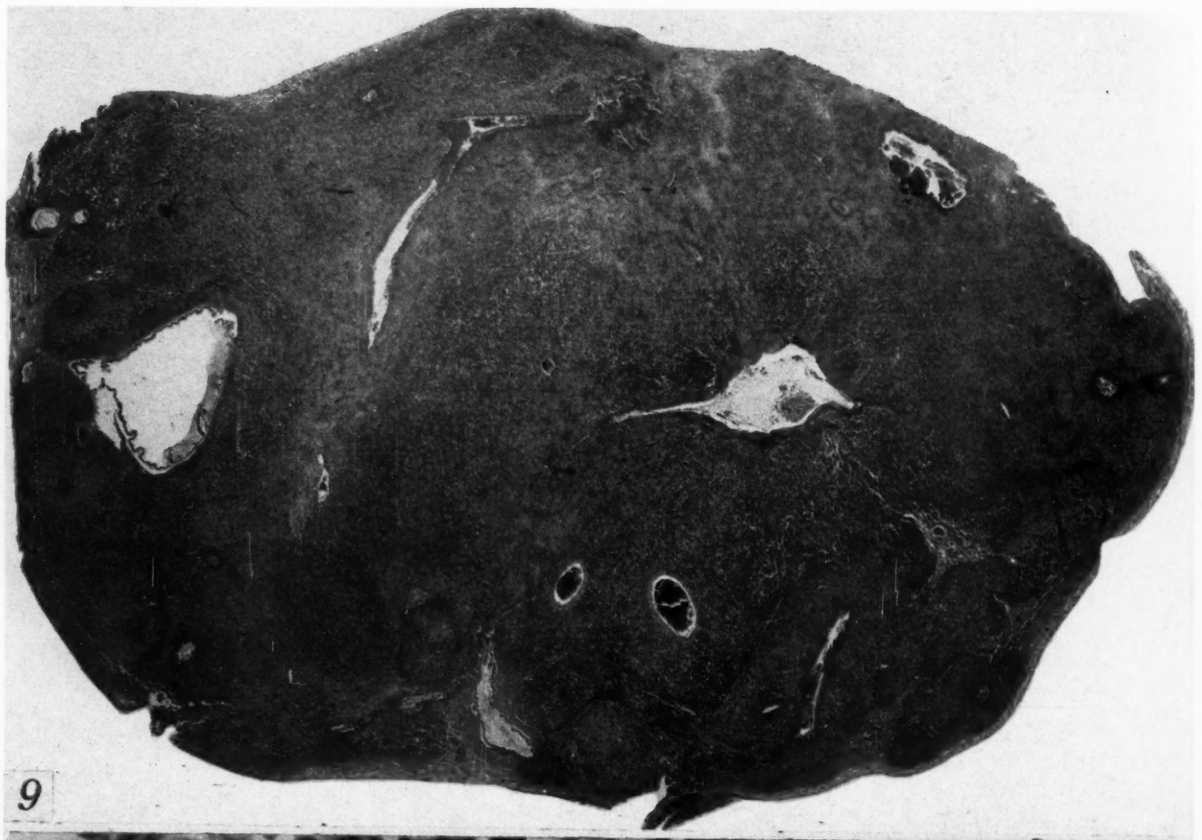


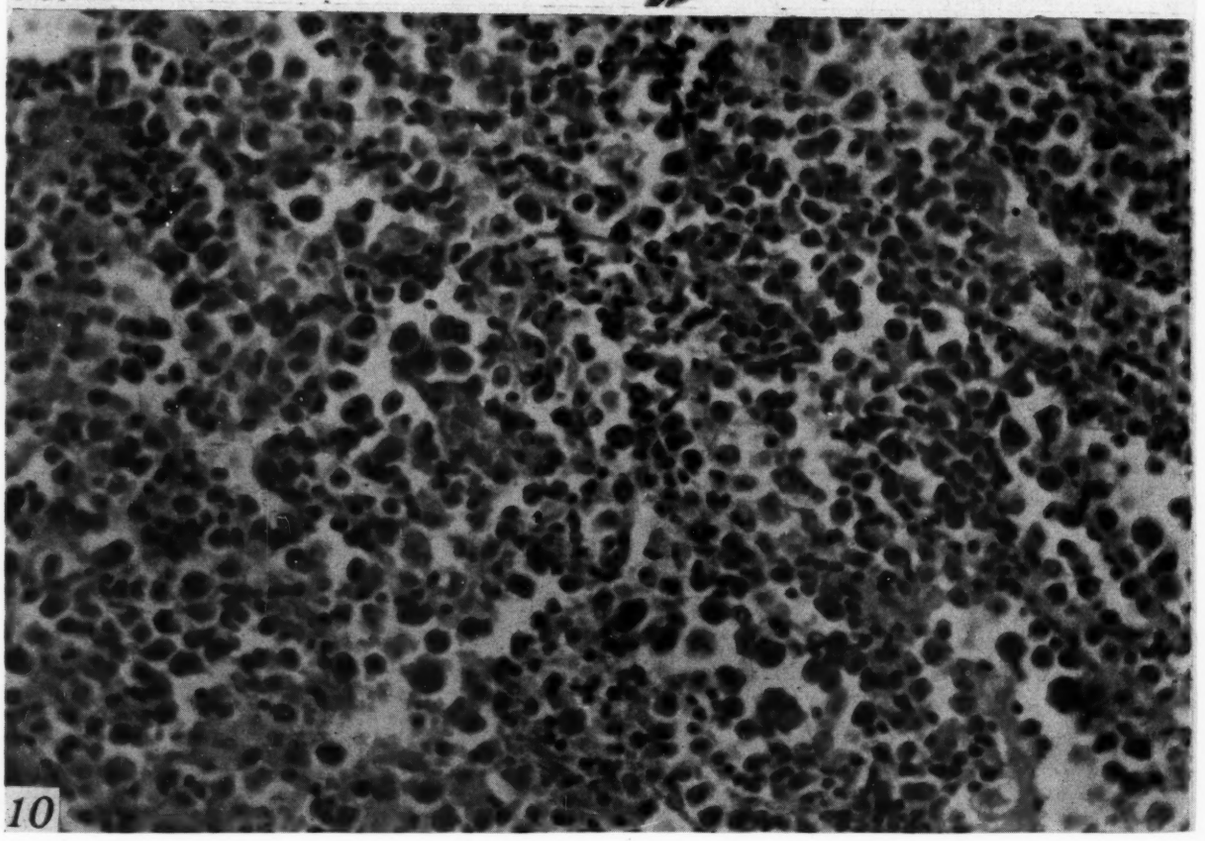
FIGS. 6–8.—LUNG OF EXPERIMENTAL RAT BH6274

FIG. 6.—Artist's sketch showing anterior (left) and posterior (right) views of lungs and mediastinum. The heart has been removed. Both lungs are filled with nodules of tumor; the lower lobes show the greatest degree of involvement. There is a mass of lymphosarcomatous tissue compressing both main bronchi. This mass of tumor was continuous with a mass in the right lung. The small fragment of tissue on the superior aspect of the hilar mass, visible in the anterior view, has invaded the right pulmonary vein and left atrium.

FIG. 7.—Replacement of normal pulmonary tissue by tumor. ×125.

FIG. 8.—Higher magnification showing malignant lymphoblasts and reticulo-endothelial cells. Many of the nuclei have large nucleoli. ×800.

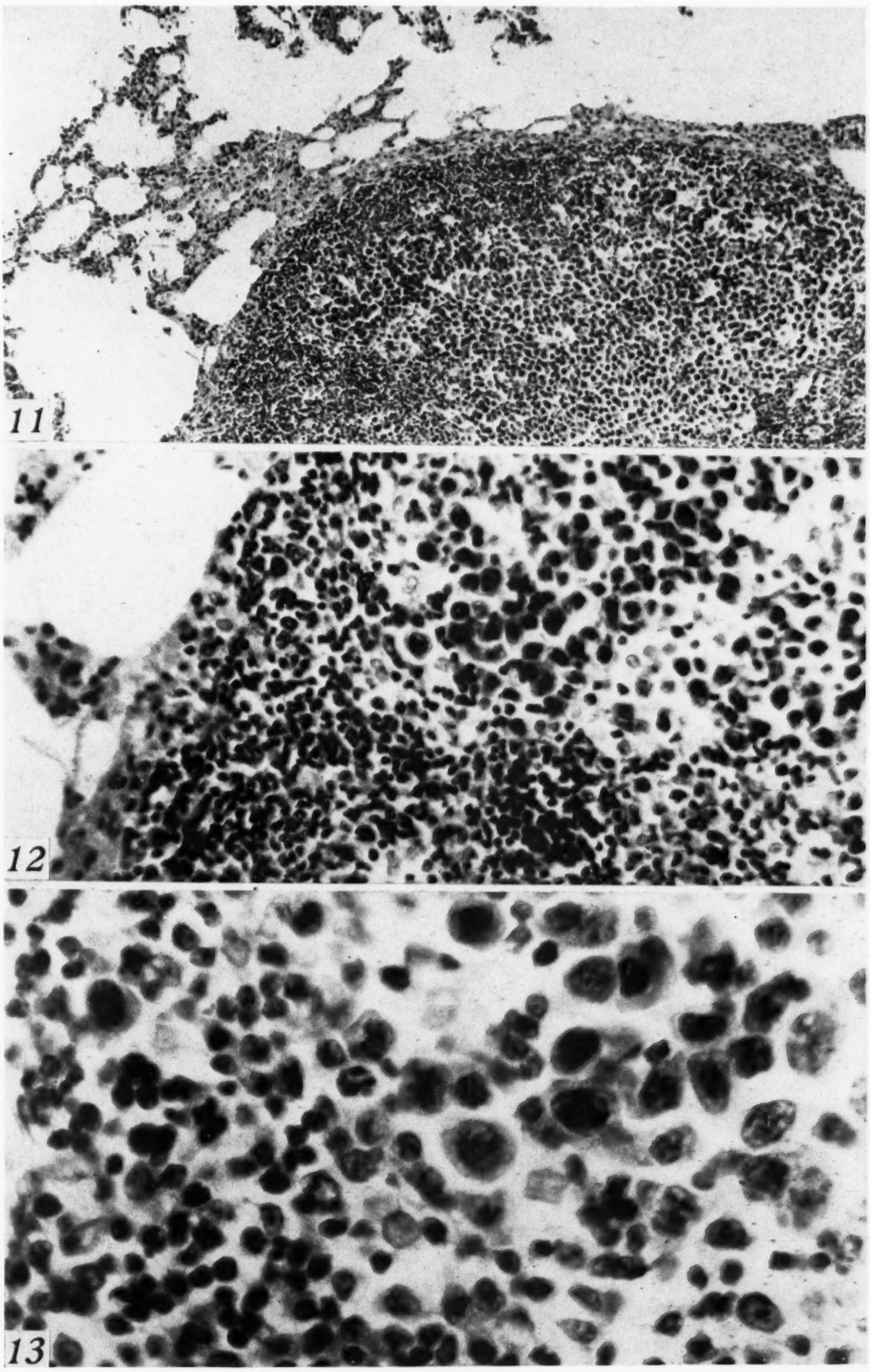




FIGS 9–10.—LUNG OF EXPERIMENTAL RAT G6553

FIG. 9.—Topographic view of cross section of right lung. There is complete replacement of pulmonary tissue by a tumorous mass greatly exceeding the original size of the organ. ×8.

FIG. 10.—Note remarkable pleomorphism of lymphosarcoma cells. ×355.

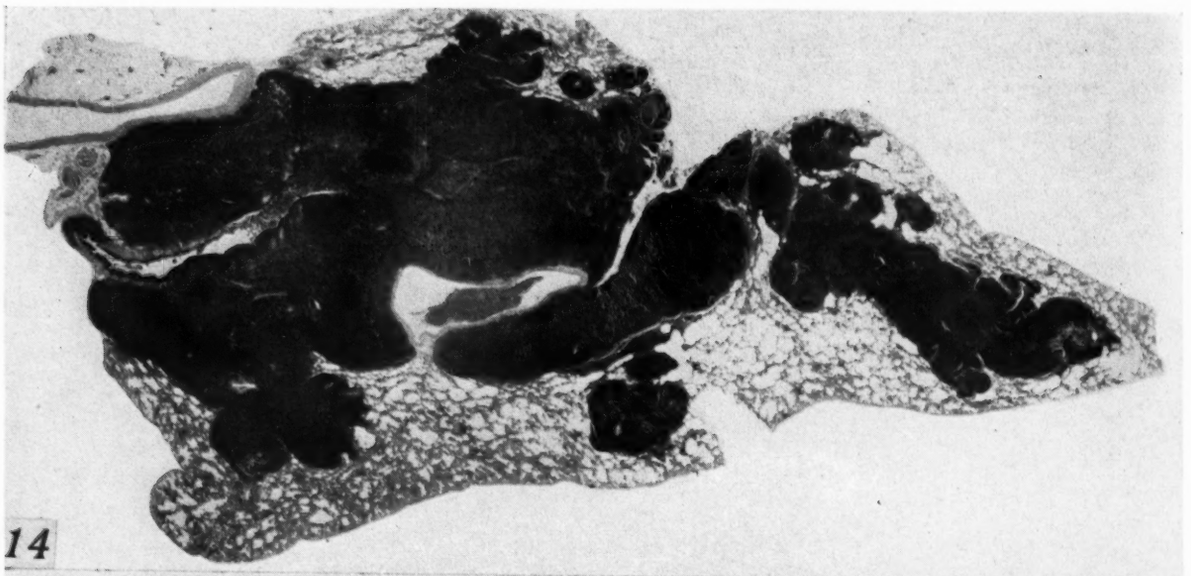


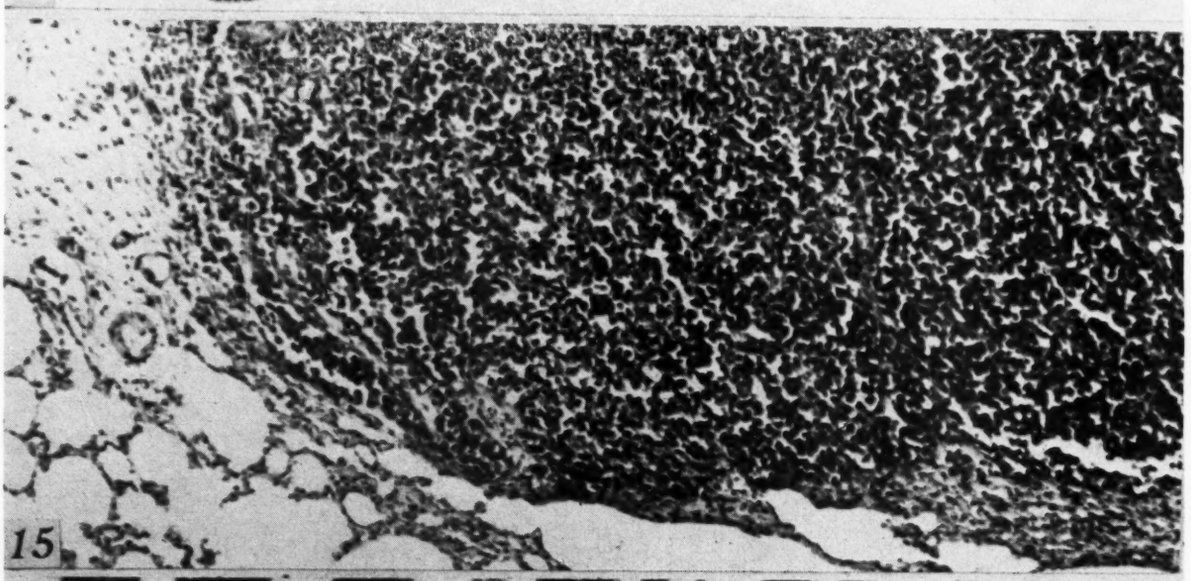
FIGS. 11–13.—LUNG OF EXPERIMENTAL RAT G6271

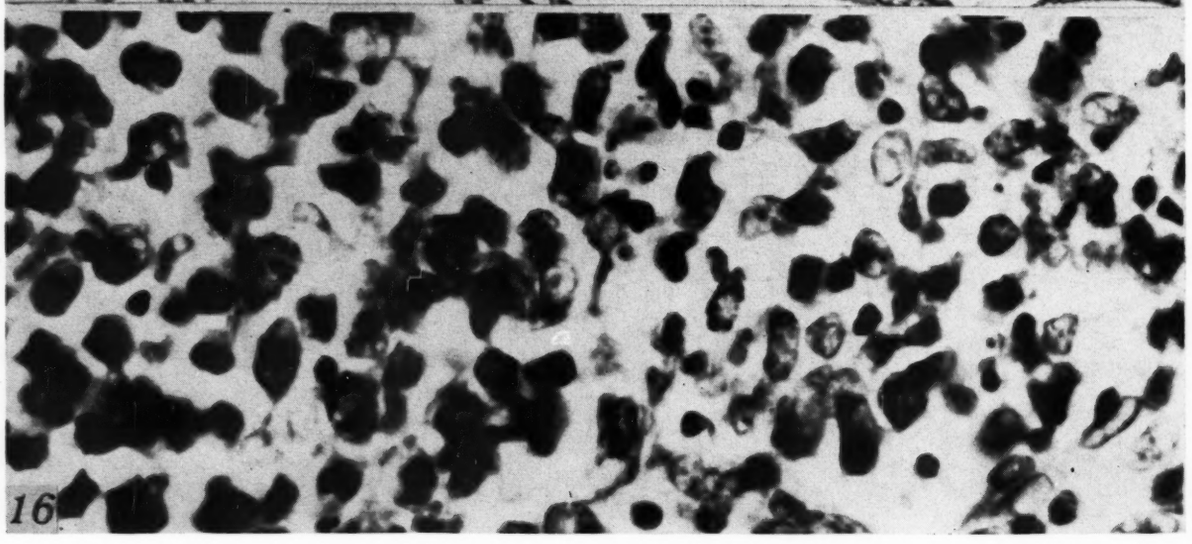
FIG. 11.—Peribronchial lymphosarcomatous transformation in the left lung. The major tumor was in the right lung. Non-neoplastic lymphocytes are seen at the periphery. ×125.

FIG. 12.—×355.

FIG. 13.—×800.







FIGS. 14–16.—LUNG OF EXPERIMENTAL RAT BH6313

FIG. 14.—Topographic view of section of left lung. The lymphosarcoma shows a peribronchial distribution. ×8.

FIG. 15.—×125.

FIG. 16.—Note the numerous bizarre cells. ×800.

All extra-thoracic lymph nodes were normal in these animals. All lymph nodes in the other rats were normal. The thymic glands were involuted in all animals treated chronically with the growth hormone. Only a small amount of fatty tissue was present in the anterior mediastinum; the thymic remnants were not weighed. The spleens of some were larger than those of the control group. However, there was no consistent increase in size of the Malpighian corpuscles. There were no neoplastic changes in the spleens.

CONTROL RATS

Lungs.—No lymphosarcomas of the lungs were present in the control rats. The lungs of these animals showed slight dilatation of some of the bronchi. There was a small amount of peribronchial lymphoid tissue (Figs. 17–20). Occasionally, the lymphoid tissue was arranged in follicles with active germinal centers. Scattered throughout the lymphoid tissue were a few macrophages which contained phagocytized particles within their cytoplasm.

In one control rat (W6523), the lower lobe of the right lung was involved by adenomatosis (Figs. 21–23). In many areas, the neoplastic cells were columnar and were arranged in large, glandular structures with frequent papillary formations. A small amount of mucus was present. The nuclei were oval and usually occupied a basal position. There were occasional mitoses. In other areas, the cells were low cuboidal and arranged in small alveolar formations and compact groups. The nuclei of these cells were small, and round or oval. Mitoses were infrequent. In many areas, the neoplastic cells were apparently supported by the pre-existing stroma of the lung. No metastases were present. This tumor was essentially similar to the pulmonary neoplasms of mice (4) and rats (3) induced with ethyl carbamate and the spontaneous pulmonary adenomatosis in the guinea pig (5).

Lymphatic tissues.—All the lymph nodes in the controls were normal except in one rat. Rat B6530, sacrificed after 378 days, had a large lobulated mass of tissue in the mesentery of the ileocaecal region (not illustrated). This tumor was composed of soft pink tissue with occasional areas of necrosis and hemorrhage. Microscopically, this tumor was formed by large, irregular cells with abundant basophilic to slightly eosinophilic cytoplasm and large nuclei. The nuclear membranes were often heavy, and the nuclear outlines were irregular. Frequently, the chromatin was present in large clumps. Nucleoli were present and often were large. Mitoses were very numerous. The cells were arranged in compact masses. Slender strands of collagen and reticulum separated groups of these cells. This tumor was considered a stem-cell (reticulo-endothelial) sarcoma arising from the ileocaecal lymph nodes and was similar to the spontaneous tumors of lymph nodes which have been previously described in the rat (1). The other lymph nodes in this animal were normal (Table 4).

The thymus glands were involuted in all control animals. The spleens were normal.

DISCUSSION

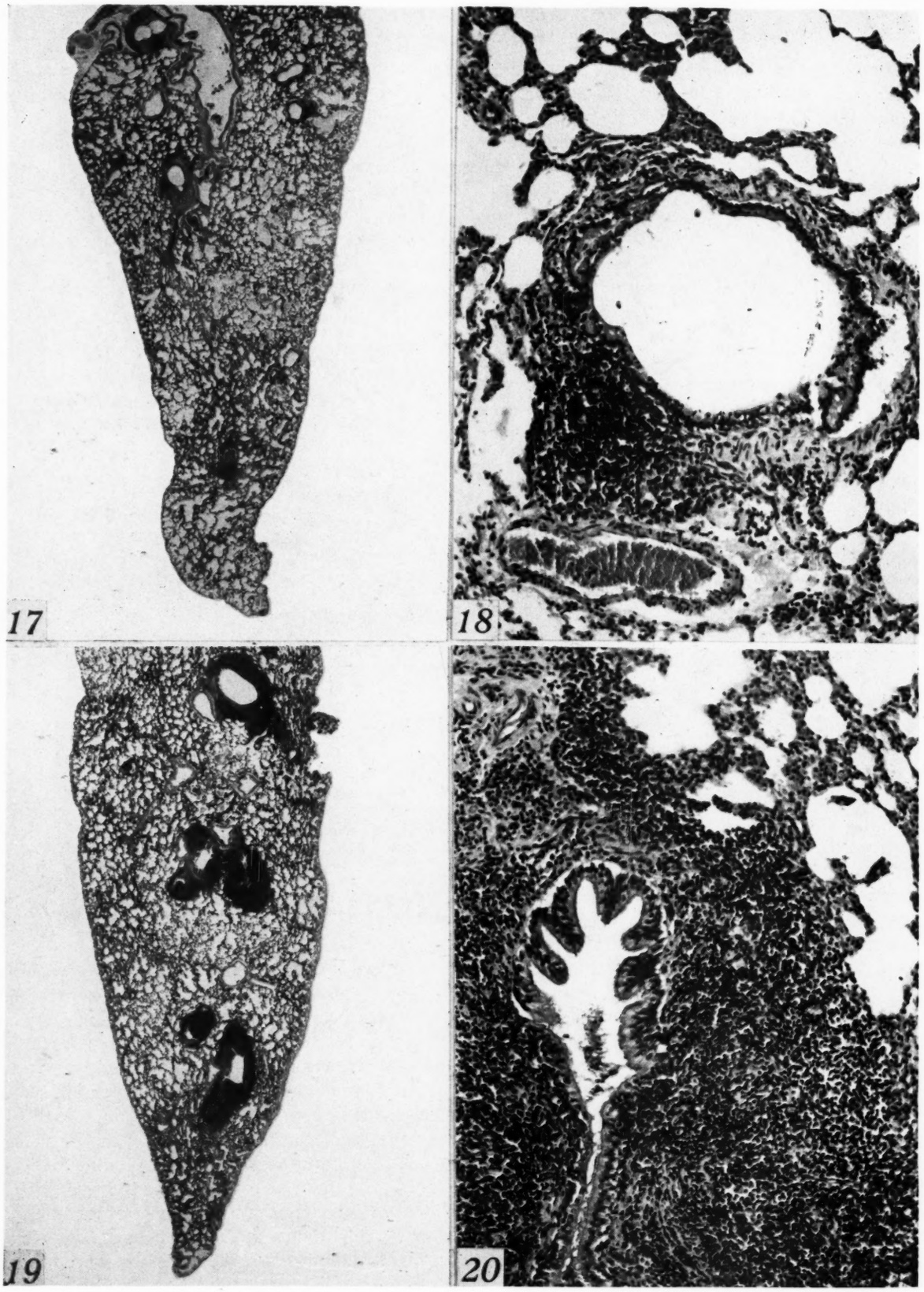
The spontaneous occurrence of lymphosarcoma of the lung has been reported by Bullock and Curtis (1). They noted that in two rats there were "polymorphous cell sarcomata of the lymphosarcoma type, which originated in and were confined to the tissues of the lung." In addition to these, there were twelve lymphosarcomas which involved the pulmonary tissues to a greater or lesser degree; and of these twelve tumors, two "may have been primary lung tumors as the major portion of each tumor was intrapulmonary."

TABLE 4

PULMONARY AND LYMPHATIC LESIONS OF CONTROL RATS INJECTED WITH ALBUMIN

Rats	Period of treatment (days)	Age at autopsy (days)	Lungs	Lymphatic tissues
B6530	378	615		Reticulo-endothelial sarcoma of ileocaecal lymph nodes
B6512	438	671		
B6400	484	722		
B6508	483	720		
W6523	483	720	Adenomatosis	
GH6442	485	722		
GH6554	485	722		
GH6357	484	722		
G6460	485	722		
GH6262	485	722		
G6268	484	723		
B6291	484	722		
BH6292	484	722		
B6492	485	722		
BH6294	485	723		

The lymphosarcomas occurring in the rats injected with growth hormone were found to be confined to the pulmonary tissues or to have involved the pulmonary tissues to a greater extent than the hilar lymph nodes. In some rats, small areas of lymphosarcomatous transformation were found in peribronchial tissue of the lung which did not contain the major mass of the lymphosarcoma. These findings lead us to believe that the lymphosarcomas occurring in the rats injected with growth hormone were primary tumors of pulmonary tissues originating in areas of peribronchial lymphoid



FIGS. 17–18.—LUNG OF CONTROL RAT BH6492

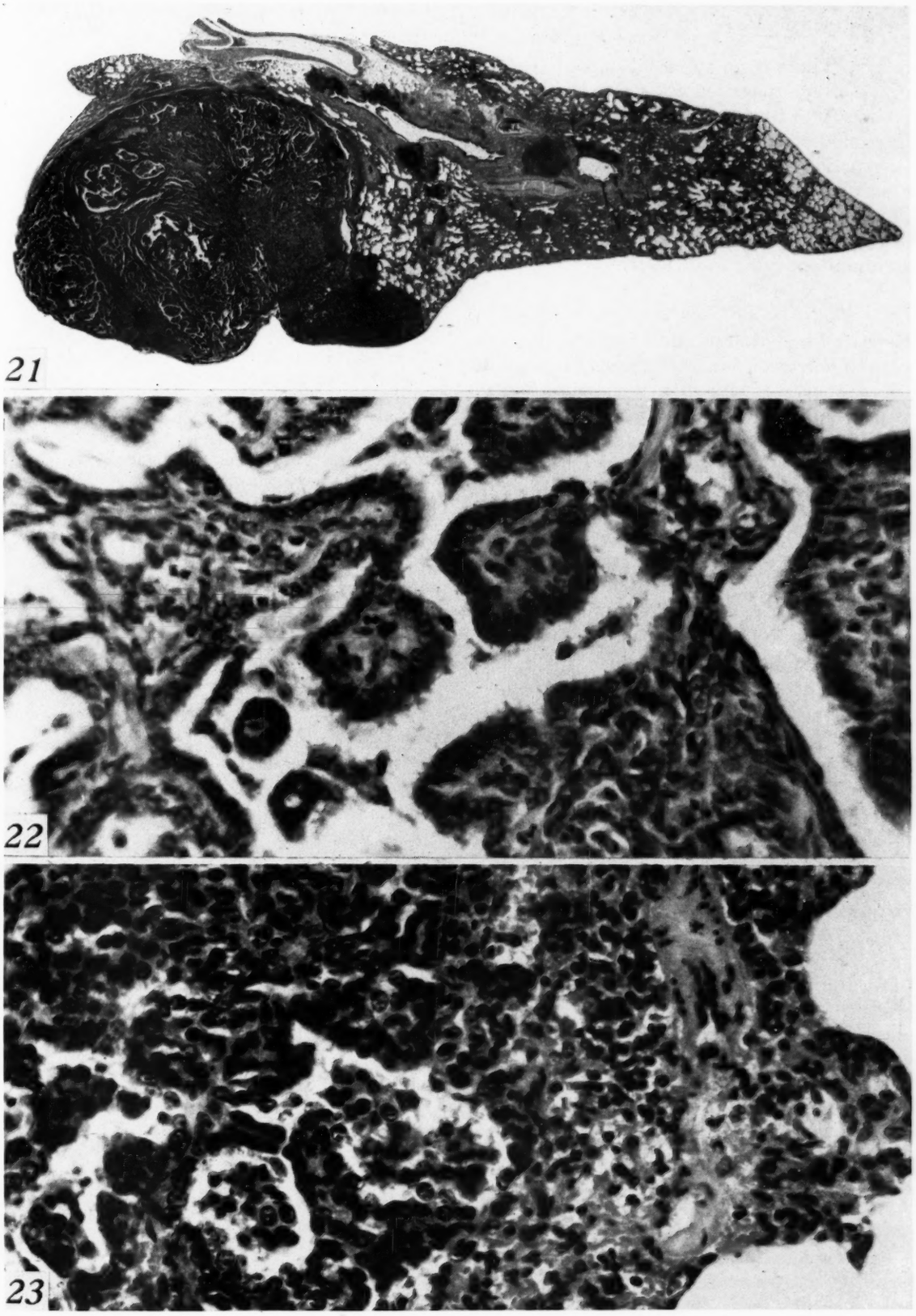
FIG. 17.—Topographic view showing the minimal amount of peribronchial lymphoid tissue occurring in control rats. ×8.

FIG. 18.—×125.

FIGS. 19–20.—LUNG OF CONTROL RAT B6400

FIG. 19.—Topographic view showing the maximal amount of peribronchial lymphoid tissue occurring in control rats. ×8.

FIG. 20.—×125.



FIGS. 21–23.—LUNG OF CONTROL RAT W6523

FIG. 21.—Topographic view of section of right lung showing adenomatosis of the lower lobe. This was the only pulmonary neoplasm occurring in the control rats. ×8.

FIG. 22.—Higher magnification of area showing papillary glandular structures formed by tall columnar cells of tumor. ×355.

FIG. 23.—Higher magnification of area showing alveolar structures and compact masses formed by cuboidal cells of tumor. ×355.

hyperplasia. There is no apparent explanation for the origin of lymphosarcomas from these areas rather than from other lymphoid tissues such as the lymph nodes, spleen, or thymus. The presence of hyperplastic peribronchial lymphoid tissue was also of considerable interest in view of the fact that a similar change could not be consistently demonstrated in any of the other organs which normally contain abundant lymphoid tissue.

SUMMARY

1. Repeated injection of adult female rats with pure growth hormone, for long periods, resulted in a continuous general body growth response similar to that previously reported.

2. Lymphosarcomas of the lung occurred in six of fifteen rats injected with pituitary growth hormone, whereas no similar tumors occurred in the control rats.

3. In all rats injected with growth hormone, there was hyperplasia of peribronchial lymphoid tissue.

4. Lymphosarcomatous transformation was observed in areas of peribronchial lymphoid hyperplasia.

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Ovary Transplanted to Spleen in Rats: The Effect of Unilateral Castration, Pregnancy, and Subsequent Castration*

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The transformations that take place in the rat ovary that has been transplanted to the spleen of a castrate rat appear to be the result of an alteration in the normal endogenous hormone pattern. The exact nature of this alteration has thus far only partially been clarified, but at least two possibilities must be considered. The first is that continuous stimulation of the intrasplenic ovary by a castrate hypophysis produces the changes; the second, that removal of estrogenic inhibition on the intrasplenic ovary permits its atypical growth. Both these effects are the result of inactivation by the liver of the endogenous hormones elaborated by the intrasplenic ovary. Other factors may also play a part.

In previous reports, we had examined the progressive changes that take place in the rat ovary that has been transplanted to the spleen after bilateral castration (4, 2), after unilateral castration (1), and after unilateral castration and subsequent pregnancy (3). In this report, the latter changes will be analyzed in more detail, with presentation of additional pertinent data.

EXPERIMENTAL FINDINGS

The rats employed in this study were of the Long-Evans strain. The animals were maintained under optimum conditions on a complete stock diet of normal composition. Food and water were available at all times. To transplant the left ovary into the spleen, a lumbar approach was used; the left ovary was removed, cleaned of fat, and separated from the tube. The spleen was then delivered into the wound, and the left ovary was placed in a pocket made by incising the splenic capsule.

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Group I (*right ovary* in situ, *left ovary in spleen*). —Included in Table 1 are the number of the animal, the age of the animal at the time of transplantation of the left ovary into the spleen, the age at autopsy, the age of the transplant, and the presence or absence of perisplenic adhesions. Thirty animals were used in this series, and the age of the transplant varied from 1 to 465 days. Perisplenic adhesions were uncommon, and most were adhesions between the spleen and pancreas, or spleen and omentum. In only one instance were there adhesions between the spleen and parietal abdominal wall.

Microscopic examination of the transplant showed that the ovary undergoes extensive necrosis immediately after transplantation; this is followed by an organizing inflammatory reaction (Fig. 1). By the fourth day after transplantation, a rim of new follicles appears at the periphery of the imflammatory zone, but these are not so numerous as in the castrate group. Subsequently, one or more corpora lutea may form, but these corpora usually are small, sharply outlined, and may have an organizing central fibrous core (Fig. 2). The developing follicles are always small, and relatively few rows of granulosa cells are present around the ova. The fibrous stroma surrounding the follicles becomes fairly prominent and contains some mononuclear inflammatory cells. As the transplant ages, it does not enlarge; often it is less than a few millimeters in diameter. Only a few follicles occur in a single field, and sometimes one contains a disintegrating ovum (Figs. 3 and 4). In transplants older than 100 days, no corpora lutea are recognizable, the follicles are always small and show little or no mitotic activity in the granulosa cells. Some fairly prominent cords of large cells that may be thecal cells are scattered in the stroma (Figs. 5 and 6). The right ovary was approximately twice normal size and composed of

large corpora lutea and scattered follicles in various stages of development.

Group II (*right ovary* in situ, *left ovary in spleen, right ovary removed later*).—Data on thirteen animals are presented in Table 2. The ovary in the spleen was permitted to undergo the atrophy described in Group I, for periods varying between 64 and 455 days. The normal right ovary was then removed and the animal sacrificed from 1 to 143 days later. On the first and second day after removal of the right ovary, the follicles in the transplant showed questionable growth (Figs. 7 and 8). As early as the fourth day, the transplant in the spleen showed definite growth activity of the follicles, which were larger and more numerous than those of the controls in Group I (Fig. 9). On the ninth day, luteinization had taken place in some follicles. On the twentieth day after removal of the normal right ovary, the transplant in one animal was composed of a cyst 1.2 cm. in diameter; this was lined by distinct large corpora lutea, and nearby there were many developing follicles. An animal

TABLE 1*

GROUP I: RIGHT OVARY *in situ*, LEFT OVARY IN SPLEEN

Tissue no.	Age at castr. and transp.	Age at autopsy (days)	Age of transp.	Adhesions	Pathologic findings of transplant
1225	82	83	1	0	Extensive necrosis and hem.; 1–3 small degenerating foll.; no C.L.
1228	82	84	2	0	Organizing inflam. reaction; rare foll.; no C.L.
1233	82	86	4	+	Organizing inflam.; ring of new foll. around periphery.
1269	82	92	10	++	Complete organization of inflam. reaction with loose C.T. replacing usual parenchyma; ring of foll. around periphery; distinct ova; no C.L.
1283	82	97	15	+	Single C.L.; several foll.; C.T. diminished.
2802	26	50	24	0	Minute transplant with minimal scarring; small foll.; no C.L.
1334	82	109	27	+	Many C.L.; developing foll.; large implant.
1979	114	151	37	0	Several C.L.; small active foll.
1980	114	151	37	0	Several foll.; minute cyst lined by org. C.L.
1981	114	151	37	+	Rare foll.; 1 organizing C.L.; many thecal cells.
1982	114	151	37	0	Several organizing C.L.; minute foll.
1983	114	151	37	+	Several minute foll. with disintegrating ova; no C.L.
1148	125	180	55	0	Minute loose C.T.; few foll. at periphery; rare ovum; no C.L.
1903	107	164	57	+	Two small org. C.L.; several minute foll. with disintegrating ova; moderate increase of interst. tissue.
1905	107	164	57	0	Minute transplant; a few small foll.; no C.L.
1907	107	164	57	0	Minute transplant; atrophic foll.; increased interst. tissue; no C.L.
1904	107	170	63	+	Minute transplant; several small foll.; disintegrating ova; no C.L.
1906	107	170	63	0	Minute transplant; several small foll.; remnant of single org. C.L.
1370	27	91	64	0	Several prominent foll. with ova; several C.L. undergoing involution.
1184	125	195	70	0	Minute transplant; central C.T. zone, small foll. at periphery; small involuting C.L.
1382	26	98	72	0	Small transplant; several foll. with ova; some minute foll.; atrophic foll.; foci of thecal cells.
1379	82	161	79	+	Small cyst with several small C.L. around periphery.
2647	31	121	90	0	Prominent transplant; many foll. with ova; no C.L.; loose C.T. stroma.
2050	48	178	130	0	Minute transplant; no C.L.; few small foll.
2710	25	175	150	0	Minute transplant; few small foll. with disintegrating ova; no C.L.
2761	26	186	160	0	Moderately prom. foll., some with distorted ova; cellular stroma; no C.L.
2126	48	220	172	+	Small transplant on surface; few foll.; no C.L.
2429	23	226	203	0	Small transplant; few foll.; no C.L.
2456	39	285	246	0	Minute transplant; rare follicles with ovum; clusters of altered follicular and thecal cells; no C.L.
2585	48	513	465	0	Minute transplant; rare atrophic foll.; stromal cells; no C.L.

* The following abbreviations are used in the tables:
C.L. = corpus luteum
foll. = follicle
inflam. = inflammation
hem. = hemorrhage
C.T. = connective tissue
org. = organizing
interst. tissue = interstitial tissue.

In each table the notations under "Adhesions" signify:
0, no adhesions; +, adhesions to pancreas, omentum, or mesentery; ++, adhesions to abdominal wall, kidney, or parietal peritoneum.

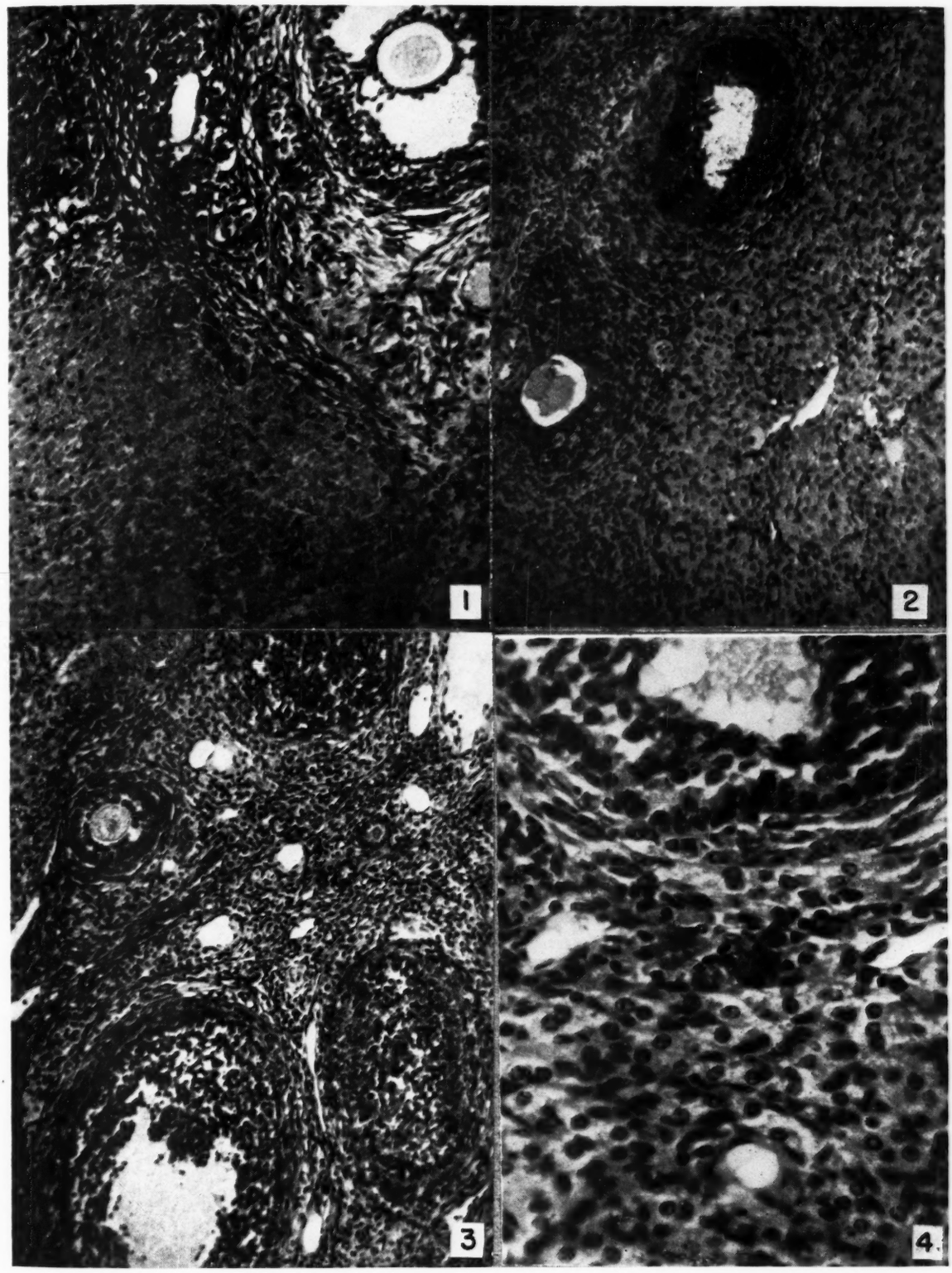

FIG. 1. Group I (No. 1228: 2 days).—The transplant has been altered by an extensive inflammatory process. The corpora lutea have undergone necrosis. Several follicles are not involved in the inflammatory reaction. ×70.

FIG. 2. Group I (No. 1370: 64 days).—The transplant is small and composed of several distinct follicles and small corpora lutea. The follicles are lined by layers of compactly arranged granulosa cells. The corpora lutea show fibrosis, particularly in the central region. ×70.

FIG. 3. Group I (No. 2710: 150 days).—The transplant contains several distorted follicles surrounded by a dense stroma. One follicle has a distinct ovum in it. The two larger follicles show alteration of the granulosa cells. The stroma contains cords of large cells that may represent thecal cells. ×70.

FIG. 4. Group I (No. 2761: 160 days).—A portion of a small follicle and surrounding stroma are shown. The follicle contains a few rows of granulosa cells and a delimiting layer of spindle-shaped cells. The large stromal cells that have a moderate amount of granular cytoplasm and a round or oval nucleus are arranged in cords and may be thecal cells. ×200.

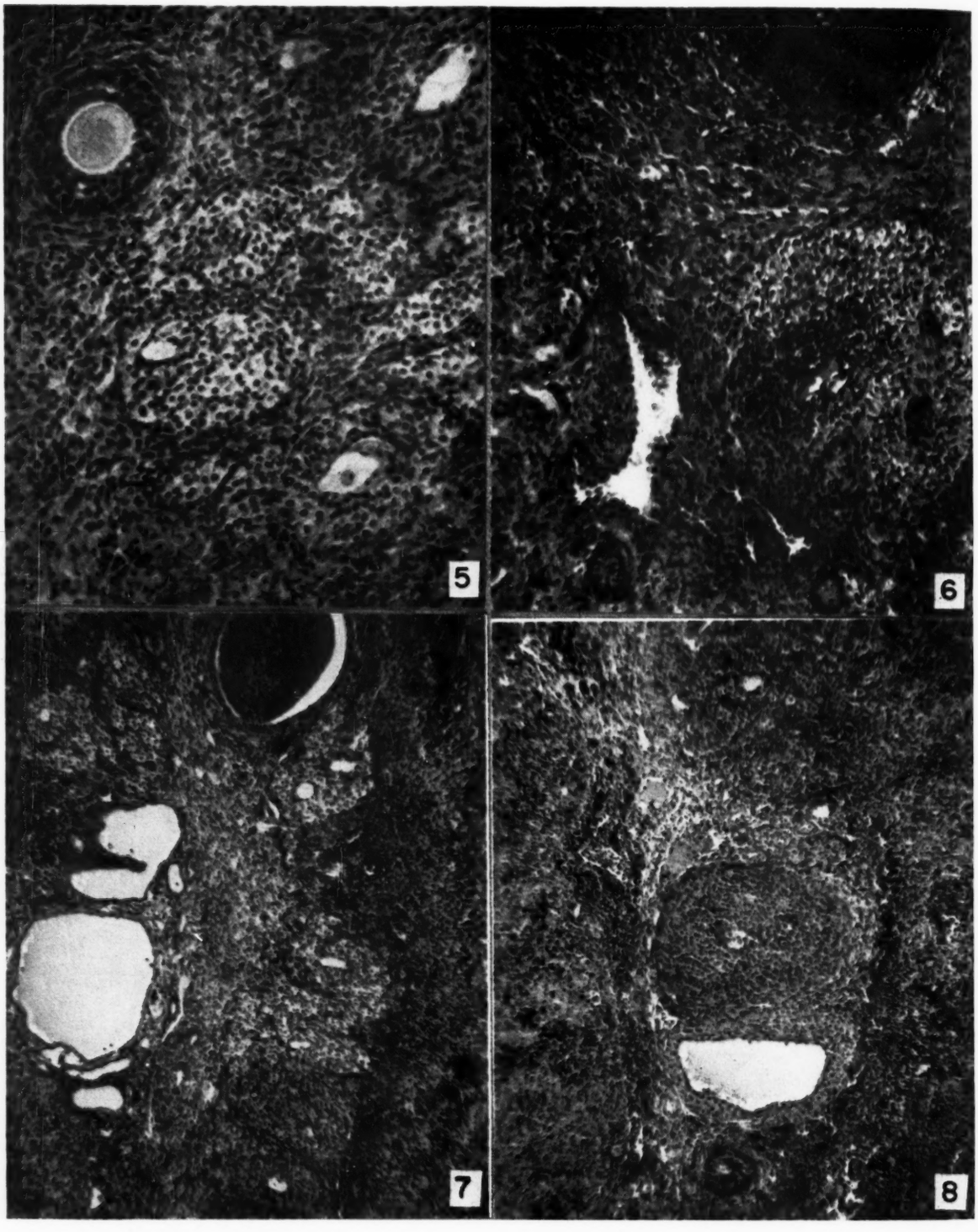

FIG. 5. Group I (No. 2456: 246 days).—The transplant is composed of dense, cellular stroma in which there are a few small follicles, one of which contains an ovum surrounded by granulosa cells. These cords of thecal-type cells make up the stroma. In some places, these cells are arranged in concentric rows so that the pattern suggests their possible origin from atretic follicles. ×100.

FIG. 6. Group I (No. 2585: 465 days).—In the dense, cellular stroma are a few distorted follicles and a rare cluster of cells that suggests a primordial follicle. A portion of a large follicle is shown and the granulosa cells are pyknotic. The stroma contains cords of thecal-type cells. ×70.

FIG. 7. Group II (No. 1384).—The transplant is surrounded by splenic pulp, and it contains one cellular follicle that may have undergone slight recent growth activity. The stroma is made up of cords and clusters of the thecal-type cells. There are several, small cystic zones that are devoid of a definitive cellular lining. ×30.

FIG. 8. Group II (No. 1383).—The transplant in the splenic pulp contains two cellular follicles, one large and one small, that are suggestive of some recent growth activity. The small cystic zone is delimited by the thecal-type cells. ×30.

sacrificed after 30 days also showed a 1-cm. cyst with corpora lutea in the wall. A solid tumor composed of many distinct corpora lutea was present in an animal examined 40 days after removal of the right ovary. The proliferation of corpora lutea in these animals was a prominent feature and closely paralleled that seen in the early stages of from 29 to 50 days, the animals were mated and became pregnant. Eight animals were sacrificed on the day the litter was born and four the next day. One or more animals were examined 9, 28, 30, 58, 60, and 90 days *post partum*. The transplants of the animals sacrificed at term or one day later were similar and, of this group of twelve animals,

TABLE 2

GROUP II: RIGHT OVARY *in situ*, LEFT OVARY IN SPLEEN, RIGHT OVARY REMOVED LATER

Tissue no.	Age at 1st operation	Atrophy in spleen	Age at 2d operation (days)	Age at autopsy	Age of transplant*	Adhesions	Pathologic findings of transplant
1384	25	199	224	225	1	+	Minute transplant composed of thecal cells; several large active foll., one with ovum; normal uterus and vagina.
1383	127	160	287	289	2	+	Minute transplant composed mainly of thecal cells; several active foll. that usually contain ova; luteinization is not evident; normal uterus and vagina.
1232†	25	143	168	172	4	+	Minute transplant composed mainly of thecal cells with several actively growing foll. that contain ova; luteinization is not evident; beginning involution of uterus and vagina.
1265	25	143	168	177	9	+	Small transplant partially replaced by two cystic foll.; one foll. undergoing luteinization; hypophysis—early castrate changes; slight atrophy of uterus; estrous vagina.
1386	22	222	244	264	20	0	1.2-cm. cyst in spleen lined by distinct C.L. that are present in the wall; many developing foll.; anestrous uterus and vagina. Hypophysis—early castrate changes.
1390	97	160	257	287	30	0	1-cm. cyst in spleen lined by distinct C.L. that are present in the wall; many developing foll.; anestrous uterus and vagina. Hypophysis—early castrate changes.
1400	24	233	257	297	40	+	Solid tumor (5.0 mm.); distinct C.L. with central fibrous zone; developing foll.; anestrous uterus and vagina; castrate hypophysis.
1401	69	223	292	332	40	++	Small transplant; few C.L. and foll.; estrous uterus and vagina.
2760	31	91	122	191	69	0	Solid tumor (437 mg.); C.L. poorly defined; many proliferating foll.; anestrous uterus.
2757	48	455	503	600	97	+	Solid tumor (400 mg.); C.L. poorly defined; many proliferating foll.; slight estrogenic activity of uterus and vagina.
2759	33	64	97	194	97	+	Solid tumor (1.2 cm.); C.L. poorly defined; many proliferating foll.; anestrous uterus and vagina; castrate hypophysis.
2927A	27	118	145	267	122	0	Solid tumor (519 mg.); C.L. poorly defined; mod. number of developing foll.; anestrous uterus; castrate hypophysis.
2987	24	91	115	258	143	0	Solid tumor (220 mg.); C.L. poorly defined; mod. number of developing foll.; anestrous uterus and vagina; castrate hypophysis.

* After removal of right ovary.

† *Left* ovary bisected; half implanted in upper pole, half in lower pole. At time of removal of *right* ovary, upper pole of spleen was removed. This transplant is minute and composed mainly of thecal cells; no distinct follicles. Right ovary: active corpora and follicles.

the tumor that develops in an animal with one ovary removed and the other transplanted to the spleen. Transformation to a luteoma did not take place in this group, however (Fig. 10).

Group III (*right ovary* in situ, *left ovary in spleen, pregnant, sacrificed* post partum).—The operative procedure performed on the 22 animals listed in Table 3 was transplantation of the left ovary to the spleen. After an interval that varied only one showed a single corpus luteum. All of them revealed active follicles with normal or slightly altered ova. The number of follicles appeared somewhat greater than in comparable animals described in Group I (Figs. 11 and 12). Four of the remaining ten animals had corpora lutea in the transplants. The degree of atrophy of the transplant increased with the duration of the *post partum* period, thus more nearly resembling the trans-

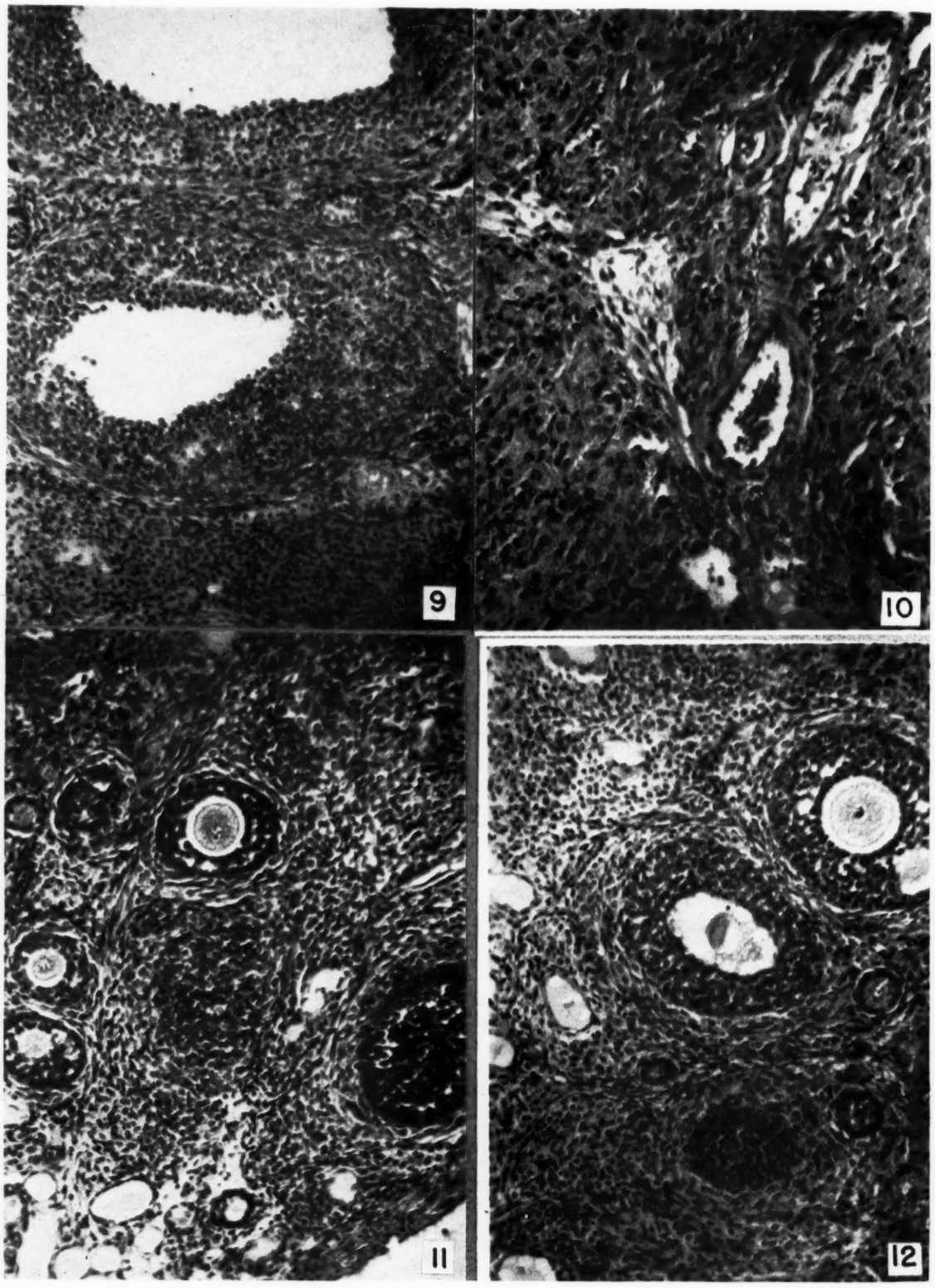

FIG. 9. Group II (No. 1232).—The transplant shows distinct growth activity characterized by the formation of two large follicles lined by many layers of granulosa cells. There are two primordial follicles. Corpora lutea have not been formed at this stage. The stromal cells are not affected. ×70.

FIG. 10. Group II (No. 2927 A).—Portions of several corpora lutea are separated by loose, fibrous septums that contain the vascular supply. Not shown are many actively growing follicles. The peculiar stroma has been replaced. ×100.

FIG. 11. Group III (No. 1389).—There are several small follicles and some contain distinct ova. The stromal cells appear increased. ×70.

FIG. 12. Group III (No. 1392).—There are two large follicles and one contains a distinct ovum. Several smaller follicles are present. The stromal cells are prominent. ×70.

plant described in Group I (Fig. 13). The absence of corpora lutea in 18 of the 22 transplants examined will receive further comment. The right ovary, which remained *in situ* throughout this experiment, underwent gross hypertrophy and was composed of large, active corpora lutea. There was little difference in the histologic appearance of the right ovaries of the animals sacrificed immediately *post partum* compared with those sacrificed later, and all closely resembled that of the right ovaries of the animals in Group I.

Group IV (*right ovary* in situ, *left ovary in spleen, pregnant, right ovary removed* post partum). —The nine animals in the group presented in Table 4 were prepared like those in Group III. The right ovary was removed either at the time of delivery or on the following day. Three animals each were sacrificed on the 10th, 30th, and 60th day *post partum*. On the 10th day, the transplants showed corpora lutea and actively growing follicles. By the 30th day, the transplants had enlarged and were composed of active follicles and large corpora lutea (Fig. 14). In one animal, an adhesion anastomosed the splenic to the systemic circulation, and growth of the transplant was impaired. At the 60th day, the transplants were larger than those ordinarily anticipated when the ovary is transplanted to the spleen of the castrate rat (Fig. 15). Again, in one animal, vascular adhesions to the systemic circulation interfered with the growth of the transplant. There is some indication in this group that the stimulation of the follicles of the transplant during pregnancy permits somewhat more rapid growth of the transplant after removal of the intact ovary.

TABLE 3

GROUP III: RIGHT OVARY *in situ*, LEFT OVARY IN SPLEEN, PREGNANT, SACRIFICED *post partum*

Tissue no.	Age at operation	Atrophy in spleen	Age at delivery (days)	Age at autopsy	Post partum period	Adhesions	Pathologic findings of transplant
1389	27	29	77	77	0	0	Active foll. and stroma; no C.L.
1398	28	35	84	84	0	++	Very small transplant containing a mod. number of sl. active small foll. with altered ova; no C.L.
1897	105	37	163	163	0	0	" " "
1898	105	41	167	167	0	+	" " "
1899	105	35	161	161	0	0	" " "
1900	105	42	168	168	0	+	" " "
1901	105	42	168	168	0	+	" " "
1902	105	36	162	162	0	+	Very small transplant with three foll.; no ova; no C.L.
1392	28	33	82	83	1	0	Many active foll. with ova; clusters of thecal cells; no C.L.
1393	27	32	82	83	1	0	" " "
1396	28	34	83	84	1	0	Many active foll. with ova; clusters of thecal cells; single C.L.
1397	28	34	83	84	1	0	Many active foll. with ova; clusters of thecal cells; no C.L.
1406	28	37	86	95	9	++	Remnant of C.L.; several foll. with some activity; distorted ova; stroma packed with thecal cells.
1407	29	37	87	96	9	0	Small transplant; fewer foll.; more stroma; no ova; no C.L.
1408	28	36	85	94	9	0	Small transplant; rare foll.; 2-mm. cysts; no ova; single C.L.
1663	27	40	88	116	28	0	Small transplant; rare foll.; 2–3 with ova; no C.L.
1664	27	40	88	116	28	+	Small transplant; rare foll.; 2–3 with ova; no C.L.
1659	27	38	86	116	30	+	Active transplant; many foll. with ova; 2 C.L.; prom. stroma.
1725	28	47	96	154	58	++	Small transplant; 3–4 foll.; 2–3 with ova; compact stroma; no C.L.
1720	27	50	98	158	60	0	Small transplant; 5–8 foll.; 4–5 ova; no C.L.
1724	28	40	89	149	60	++	Small transplant; 2–3 foll.; 2–3 with ova; no C.L.
2775	24	47	92	182	90	0	Small transplant; rare foll.; remnant of C.L.

DISCUSSION

After transplantation of one ovary to the spleen of a castrate rat, a constant and regular sequence of events takes place in the transplanted ovary. The latter first undergoes a necrotizing inflammatory reaction immediately after the operative procedure. This reaction subsides in a few days, and a group of small developing follicles is distinguishable as the only surviving component of the original ovarian tissue. These follicles grow actively and luteinize, while, at the same time, new follicles appear. The corpora lutea become large and distinct and do not involute. In a period of a few months, the transplant grows into a large, encapsulated mass of corpora lutea, with a variable number of developing follicles scattered

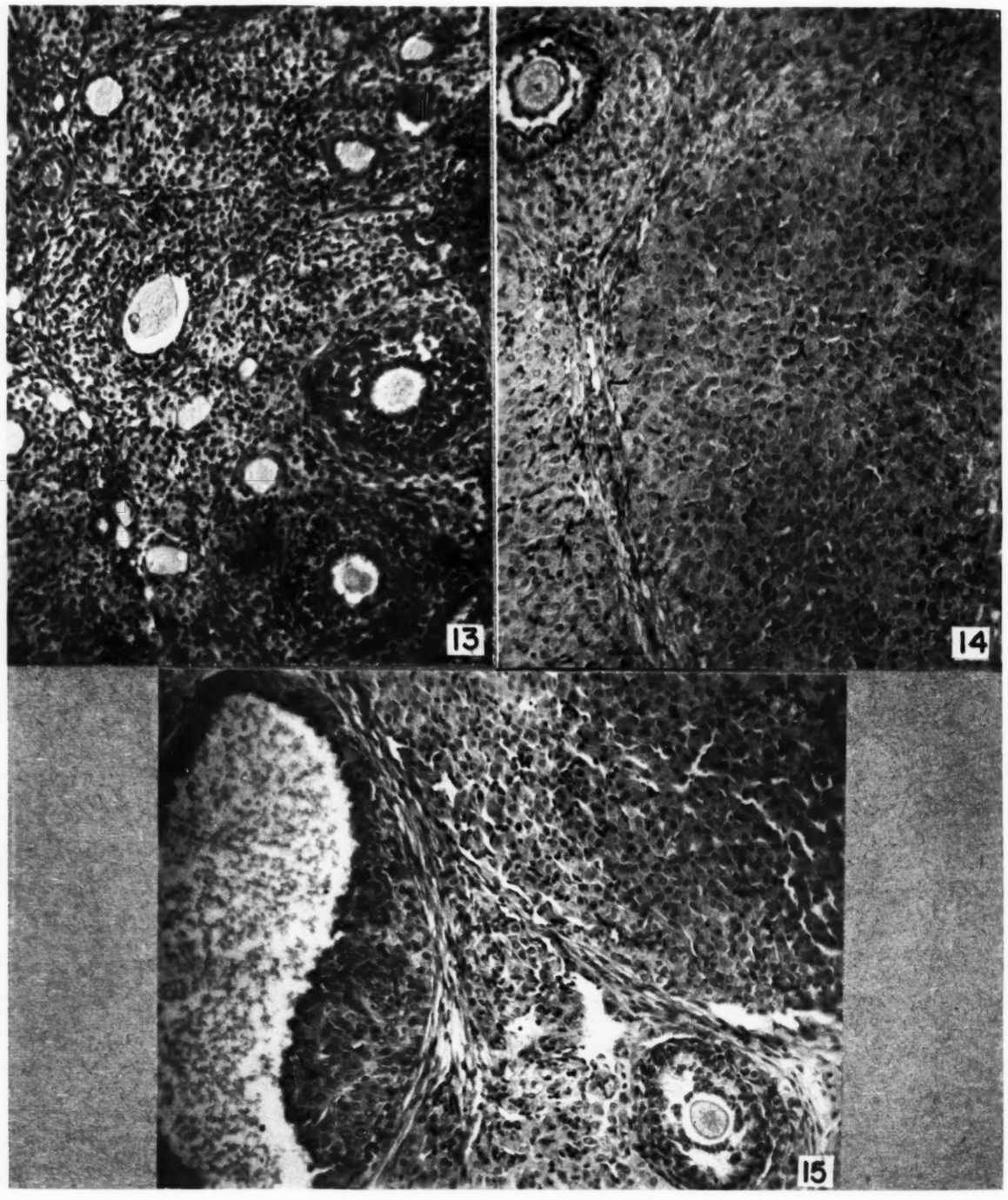

FIG. 13. Group III (No. 1407).—Two follicles are shown, and there is one small follicle with a disintegrating ovum. The stromal cells are prominent and often arranged in groups. In some places these stromal cells suggest that they are the result of involution of follicular cells. ×70.

FIG. 14.—Group IV (No. 1661).—There are segments of three large corpora lutea and one growing follicle The latter contains a distinct ovum. The corpora are separated by fine strands of connective tissue. ×100.

FIG. 15. Group IV (No. 1723).—Parts of two large corpora lutea and some smaller masses of luteal cells are present. The follicle is surrounded by luteal tissue. One corpus luteum has a cystic zone. Strands of connective tissue separate the corpora. ×100.

among them. About 5 months after transplantation, a growing nest of luteal cells may first be noted that expands and pushes aside the corpora lutea. With the appearance of the luteoma, developing follicles no longer are found in the transplant. The proliferating cells of the luteoma resemble the luteal cells of the corpora in size and shape, but the pattern of the luteoma is one of solid masses of cells or anastomosing cords and plugs surrounded by a network of endothelial-lined vascular spaces. As the luteoma grows, the corpora are compressed and ultimately disappear. In some instances, this is the final pattern, but in many tumors isolated nests of smaller cells appear that have been interpreted as granulosa cells. The granulosa-cell tumors do not occur in transplants that are younger than 10 months. The granulosa cells may remain as isolated masses, or may entirely replace the luteoma. They may show a wide variety of patterns. The cells are usually smaller than the luteal cells, and they have a compact hyperchromatic nucleus and scant cytoplasm. The intense vascularization of the tumors is an outstanding feature and undoubtedly is related to their hormonal activity. In the 46 luteomas that were examined 157 days after the transplantation of the ovary, granulosa-cell zones were present in 24 (2). Of the tumor-bearing animals, 28 showed cornification of the vagina and uterus and chromophobic alteration of the anterior lobe of the hypophysis, indicating that at a certain stage in the growth of some of the tumors, some form of estrogen, probably derived from the tumor, escaped inactivation by the liver and stimulated the susceptible end organs.

The luteoma and the granulosa-cell tumors do not appear in a transplant that has developed large vascular adhesions to the systemic circulation. The tumors do not occur in an ovary transplanted to the kidney. These findings indicate that the inactivation or alteration by the liver of the hormones elaborated by the transplanted ovary in the spleen is necessary for the growth of the tumors, at least to a certain stage.

In view of these findings, the failure of the intrasplenic ovary to grow when one ovary remains in its normal position is striking. It may be that the gonadotrophins elaborated by the hypophysis are insufficient to stimulate the ectopic ovary, and thus the latter undergoes atrophy. However, at the same time, the gonadotrophins are sufficient to stimulate the normally situated ovary to the same size it would have reached if the animal were a unilateral castrate. Furthermore, the gonadotrophins stimulate the latter ovary to form normal follicles, ova, and corpora lutea; normal estrous cycles occur, and the animals can become pregnant. It is noteworthy that when the intrasplenic

TABLE 4

GROUP IV: RIGHT OVARY *in situ*, LEFT OVARY IN SPLEEN, PREGNANT, RIGHT OVARY REMOVED *post partum*

Tissue no.	Age at 1st operation	Atrophy in spleen	Age at delivery	Age at 2nd operation	Age at autopsy	Post partum period	Adhesions	Pathologic findings of transplant
			(days)					
1403	29	36	86	86	96	10	0	Many minute cysts lined by luteal cells; few foll. and C.L.; early anestrous uterus and vagina.
1404	27	38	86	86	96	10	0	Prom. C.L. and foll. with prolif. of new foll.; anestrous uterus and vagina; hypophysis—many eosinophils; some vacuolated cells and chromophobes.
1405	27	38	86	86	96	10	+	Foll. and small C.L. showing activity; early anestrous uterus and vagina.
1660	29	34	84	85	115	30	++	Scattered developing foll.; small C.L. showing central fibrosis; cornified vagina, estrous uterus; hypophysis—chromophobe hyperplasia.
1661	27	36	84	85	115	30	+	Large transplant with active foll. and large C.L.; castrate hypophysis; anestrous uterus and vagina.
1662	28	35	84	85	115	30	+	Large transplant with active foll. and large C.L.; anestrous uterus and vagina; castrate hypophysis.
1721	29	34	84	85	145	60	++	Small transplant, active foll.; some C.L. show organization and cysts; large active uterus and cornified vagina.
1722	28	35	84	85	145	60	++	Prom. transplant (10×7×6 mm.); active foll.; some C.L. show organization or cyst formation; anestrous uterus and vagina; castrate hypophysis.
1723	28	35	84	85	145	60	+	Large transplant (12×6×6 mm.); active foll. and C.L.; anestrous uterus and vagina; castrate hypophysis.

ovary atrophies under these conditions, it simulates the degree of atrophy that follows hypophysectomy. Following removal of the normally situated ovary, the intrasplenic ovary at once shows developing follicles followed by corpora lutea, thus indicating that the growth potentialities are always present.

When animals with one ovary intact and the other in the spleen become pregnant, another puzzling phenomenon occurs. Instead of the luteinization to be anticipated in the transplant at the termination of the pregnancy, the intrasplenic ovary, with only an occasional exception, shows an increase in the number of follicles which appear more cellular than those in the nonpregnant controls. It is surprising that the gonadotrophins, hypophyseal or chorionic, are insufficient to produce corpora lutea in the intrasplenic ovary while they are adequate to maintain the corpora of pregnancy in the normally situated ovary. Instead, there is a moderate increase and enlargement of the follicles of the intrasplenic ovary, an unexpected action for the types of gonadotrophin normally secreted during pregnancy.

SUMMARY

A regular sequence of events occurs after one ovary has been transplanted into the spleen of a castrate rat. After the initial inflammatory reaction has subsided, there is a constant development of new follicles which luteinize. These corpora lutea do not involute, and from them, after 5 months or more, a luteoma appears. Subsequently, a granulosa-cell tumor may replace the luteoma. If an ovary is transplanted into the spleen of a unilaterally castrate rat, the intrasplenic ovary atrophies. The growth potentialities of this atrophic intrasplenic ovary become evident as soon as the normal ovary is excised. The effect of pregnancy on the intrasplenic ovary of unilateral castrates is to produce active follicular growth usually without the formation of corpora lutea. Following termination of the pregnancy, the intrasplenic ovary atrophies; however, simultaneous termination of the pregnancy and castration produce active growth in the intrasplenic ovary.

The changes that take place in the ovary transplanted to the spleen of a unilaterally castrated rat were examined in 30 animals at intervals varying from 1 to 465 days. The transplant undergoes a progressive decrease in size, rarely contains corpora lutea, the number of follicles decreases, and the stroma contains cords of large cells that resemble thecal cells. Despite this atrophy, if the normally situated ovary is removed, the transplant is capable of growth characterized by the continuous formation of many follicles which undergo luteinization. These findings were demonstrated in thirteen animals. The effect of pregnancy on the intrasplenic ovary in the unilateral castrate was examined in 22 animals. In twelve that were sacrificed at the time of delivery or the next day, the transplant revealed many small follicles; in only one was there a corpus luteum. The remainder were examined at intervals after the delivery, and the transplant decreased in size, contained fewer and smaller follicles, and in three there were remnants of one or two corpora lutea. Again, removal of the normally castrated ovary at the termination of pregnancy permitted the intrasplenic ovary to undergo a somewhat accentuated follicular and luteal growth.

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The Effect of Added Dietary Tryptophane on the Occurrence of Diethylstilbestrol-induced Mammary Cancer in Rats*

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A comparative study by White, White, and Mider (8) of the effect of a restriction of cystine, lysine, and tryptophane in the diet of dba mice showed that the development of methylcholanthrene-induced leukemia was inhibited by a restriction in cystine but was unaffected by a restriction in the essential amino acids, lysine, and tryptophane. A previous study by White and Andervont (7) showed that the restriction of dietary cystine completely inhibited the formation of spontaneous mammary cancer in C3H mice and that mice on a high-cystine diet developed mammary cancer in the same proportion as C3H mice on a Purina Dog Chow diet. The average latent period for the tumors in the mice on the high-cystine diet was significantly shorter than that for the tumors in the control groups. No mammary cancers were observed in the 45 C3H mice on the low-cystine diet, although all survived the average latent period for the occurrence of these tumors and 36 survived for 22 months. Of 42 mice on the high-cystine diet, 41 developed mammary tumors in an average of 8.4 months, while 341 of 350 C3H mice on Purina Dog Chow developed mammary tumors in an average of 10.4 months. The percentage of tumor mice in the two latter groups was similar, but the difference in average latent period of 2.07 ± 0.23 months is certainly significant and equivalent to one-fifth the average latent period observed for the tumors of the control group.

In a later report, White and White (6) showed that the implantation of diethylstilbestrol pellets partially compensated for the effect of the low-cystine diet, indicating that, in part, the inhibition of the mammary tumors resulted from lack of hormonal stimulation in the mice on the deficient diet. In this experiment, 17 of the 40 treated mice on the low-cystine diet developed mammary tumors in an average of 11.18 months, while 23 of 27 mice on Purina Dog Chow developed mammary tumors in an average of 9.13 months and 23 of 28 mice on the high-cystine diet developed mammary tumors in an average of 8.43 months. The average latent period was significantly longer in the mice on the low-cystine diet than in the mice of the two other groups and was shortest in the mice on the high-cystine diet, although the difference between the latter group and the controls does not appear to be significant. These experiments indicate that a reduction below the optimum dietary intake of cystine inhibits mammary cancer formation and the occurrence of methylcholanthrene-induced leukemia, and that increased dietary cystine has an accelerating action on the development of mammary cancer.

The present experiments were undertaken for the purpose of exploring the effects of deficiencies and excesses of tryptophane on the occurrence of diethylstilbestrol-induced mammary cancer in rats. They included five experimental diets containing, respectively, 0.10, 0.14, 1.0, 1.4, and 4.3 per cent DL-tryptophane. No significant data were obtained from the first group because the rats failed to survive the average latent period for the induction of these tumors. Comparative data are not yet available for the groups which received 0.14 and 1.0 per cent tryptophane, but completed data for the two groups which received the greatest increment of tryptophane seemed of sufficient interest to report at this time.

MATERIAL AND METHODS

Pedigreed female rats of A×C line 9935 (2) between 4 and 5 months of age were used for the experiments. Cholesterol pellets containing 4–6 mg. of diethylstilbestrol[1] were implanted subcutaneously in the scapular region, to maintain a continuous state of hyperestrinism. A previous re-

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[1] Supplied through the courtesy of the late Dr. D. F. Robertson of Merck & Co., Rahway, N.J.

port (1) showed that 75 per cent of similarly treated female rats of this line, on an average daily consumption of 34 calories of a synthetic diet, developed multiple mammary cancers in 10 months.

In the present experiment, each rat was housed in an individual cage, with free access to water. The daily portion of 7 gm. of diet was weighed out and presented in a food cup, to which was added the daily supplement of crystalline vitamins. The diet of 6.5 per cent fat content (1). An attempt was made to recover and weigh all food that was spilled. The proportion of protein was chosen on the basis of McCoy's (3) finding that approximately 25 per cent was optimum for lactation.

Each rat was weighed and inspected for mammary cancers once a week. At death, post-mortem examination included a detailed description of every visible tumor, gross sectioning of lungs and mammary glands, and the inspection and weight of the liver, kidneys, adrenals, pituitary, and sex glands. Representative sections of each of these tissues and organs were preserved for microscopic examination. All cancer foci that could be identified were tabulated.

TABLE 1

THE NUMBER OF RATS IN EACH GROUP, THEIR AVERAGE INITIAL BODY WEIGHT, AND DAILY FOOD CONSUMPTION

Group	Number of rats	Body weight (gm.)	Daily ration (gm.)	Daily ration (calories)
Diet 3	12	162	6.9	32
Diet 7	12	153	7.0	33
Diet 5	15	160	6.8	32

RESULTS

The number of rats in each group, their average initial body weight, and daily food consumption are shown in Table 1. The 12 rats on Diet 7, the control diet, consumed all the food that was offered them, while those on Diets 3 and 5, with the added tryptophane, averaged 32 calories per rat per day, or 1 calorie less per day than those on the control diet.

TABLE 2

THE NUMBER OF RATS WHICH SURVIVED FOR AT LEAST 160 DAYS, THEIR AVERAGE POST-MORTEM BODY WEIGHTS IN GRAMS, AND THE PERCENTAGE WEIGHTS OF SOME OF THE ORGANS

Group	Number of rats	Average body wt.	Organ weights in per cent of body weight					
			Liver	Kidney	Adrenal	Pituitary	Ovary	Uterus
Diet 3	12	121	5.5	1.4	.05	.06	.05	.66
Diet 7	12	117	4.4	1.2	.05	.06	.04	.65
Diet 5	15	99	5.4	1.5	.05	.03	.04	.75

three diets differed only in the composition of the protein as follows:

	Diet 7 (per cent)	Diet 3 (per cent)	Diet 5 (per cent)
Casein	26.0	0	0
Tryptophane-free casein hydrolysate[2]	0	25.0	22.0
Salt mixture	4.0	4.0	4.0
Cellu flour	2.0	2.0	2.0
Dextrin	54.0	54.0	54.0
Crisco	15.0	15.0	15.0
Halibut liver oil	0.4	0.4	0.4
DL-tryptophane	0	1.4	4.3

plus the following crystalline vitamin[3] supplement per kilogram:

Thiamin	4 mg.
Riboflavin	8 mg.
Pyridoxine HCl	4 mg.
Niacin	4 mg.
Calcium pantothenate	20 mg.
Choline HCl	2,000 mg.
Alpha-tocopherol	150 mg.

Each gram of diet was equivalent to 4.7 calories, and the daily portion fed was 33 calories, a little less than the *ad libitum* consumption of a synthetic

[2] Supplied through the courtesy of Dr. Charles F. Kade of the Sterling-Winthrop Research Institute, Rensselaer, N.Y.

[3] Supplied through the courtesy of Dr. R. C. Pogge of Merck & Co., Rahway, N. J.

A decrease in body weight as shown by the average post-mortem weights in Table 2, is characteristic of diethylstilbestrol-treated rats. However, the rats on Diet 5 with the greatest increment of tryptophane lost proportionately more than the rats of the two other groups. Their caloric consumption was essentially similar, so they must have failed to utilize some of the ingested food. There were no significant differences in the organs which were weighed as shown by their percentage weights in Table 2, except in the case of the pituitary. The average pituitary weights were 70, 76, and 29 mg., respectively, for the rats on Diets 3, 7, and 5. The respective percentage weights were 0.06, 0.06, and 0.03. If size of organ is correlated with function or physiological demand, the rats on the high-tryptophane diet had less pituitary hormone stimulation than the rats of the two other groups.

The average survival in days and the number

and percentage of induced mammary cancers are given in Table 3. The individual survival record, time of observation of the first mammary cancer, and number of induced cancers for each rat are shown graphically in Figure 1. The twelve rats on Diet 3 all developed multiple mammary cancers in an average of 316 ± 5.7 days, while nine of the twelve rats on the control or 26 per cent casein diet developed multiple mammary cancers in an average of 363 ± 8.6 days. The number of rats in each group is too small for the difference in per cent of induced tumors, as shown in Figure 2, to be significant; but the difference of 47 ± 10 days in the mean latent period is certainly significant. This is shown graphically in Figure 3. Table 3 also shows that 79 gross mammary cancers—including with these the cancers identified by microscopic examination of the mammary tissue, a total of 194 cancer foci—were observed in the rats on Diet 3; while 51 gross cancers, and a total of 133 cancer foci, were identified in the rats of Diet 7. The average weight of mammary cancers per rat was 13.2 in the former group and 10.7 in the latter group, while the individual tumors averaged 2.0 gm. in the rats on Diet 3 and 1.7 gm. in the rats on Diet 7. All these differences seem to indicate that the substitution of approximately 1 per cent of DL-tryptophane for casein, in an otherwise similar diet, increased the susceptibility of these rats in induced mammary cancer.

These same charts and Table 3 show, conversely, that the substitution of as much as 4 per cent DL-tryptophane for a similar amount of casein decreased the number and percentage of induced mammary cancers and significantly prolonged the average latent period. In this group 9, or 60 per cent, of 15 rats developed 31 macroscopic and a total of 109 macroscopic and microscopic induced mammary cancers in an average of 399 ± 7.5 days. The average weight of mammary cancer per cancer rat was only 5.1 gm. compared with 13 gm. for cancers in rats on Diet 3, and the individual tumors averaged 1.5 gm. compared with 2.0 gm. The effect of the increased proportion of tryptophane seems to be analogous to that of caloric restriction, as shown in a previous report (1), but disproportionately great for the loss of approximately 3 per cent of unusable protein. The caloric intake was similar in the three groups of rats, but, obviously, there was a difference in assimilation,

TABLE 3

THE AVERAGE SURVIVAL IN DAYS, THE NUMBER AND PERCENTAGE OF RATS IN EACH GROUP WITH MULTIPLE MAMMARY CANCERS, THE NUMBER OF MACROSCOPIC CANCERS, AND THE TOTAL NUMBER OF CANCER FOCI OBSERVED, THE AVERAGE WEIGHT OF CANCER PER RAT, AND THE MINIMUM AND MEAN LATENT PERIOD IN DAYS

Group	Number of rats	Average survival	Rats with ca.		Number of cas.		Ave. wt. of cas.	Minimum latent period	Mean latent period
		(days)	(No.)	(per cent)	Macroscopic	Total	(gm.)		
Diet 3	12	361	12	100	79	194	13.2	209	316 ± 5.7
Diet 7	12	433	9	75	51	133	10.7	163	363 ± 8.6
Diet 5	15	399	9	60	31	109	5.1	288	399 ± 7.5

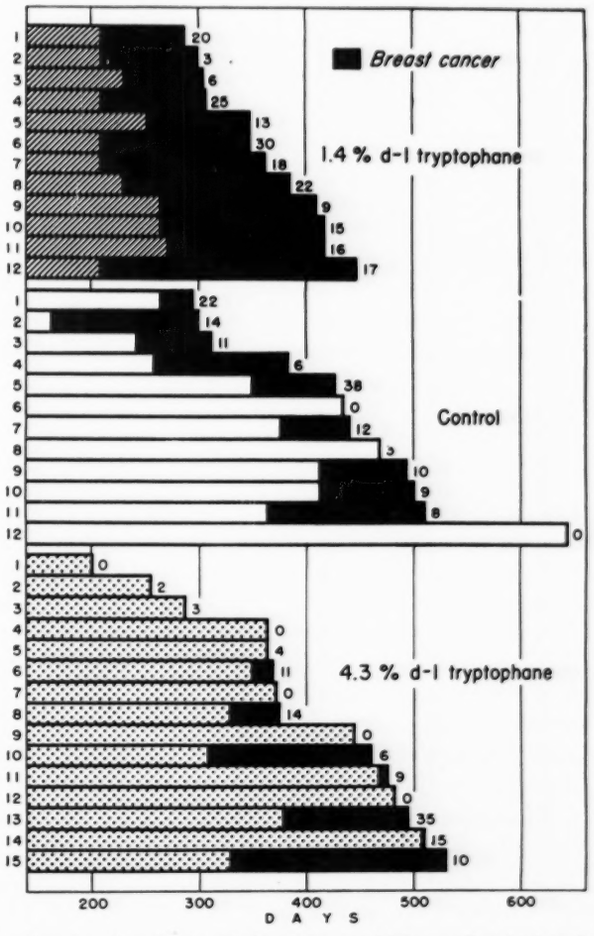

FIG. 1.—Survival period and tumor history of rats of each group. (Each rat is represented by a bar, the length of which indicates the period of survival; the blackened area represents the time elapsed after observation of the first mammary cancer; and the number at the right of the bar represents the total number of mammary cancer foci identified in the post-mortem study.)

for the rats on Diet 5 weighed nearly 20 per cent less than the rats of the two other groups.

Tannenbaum and Silverstone (4, 5) have recently concluded, on the basis of experiments with spontaneous mammary and lung cancers and induced skin cancers in mice, that the effect brought about by caloric restriction is a function of the low body weight of the animals. That is, the actual caloric intake or metabolic turnover is not as important in the genesis of these tumors as the balance struck between caloric intake and utilization. This might explain the effect observed here and the previously reported lack of effect from the increased consumption of a high-fat diet (1), but this theory is not substantiated by the results of a forthcoming study of acetylaminofluorene-induced liver and bladder cancer in rats on similar diets. In this study, rats with low body weights do not show a proportionate reduction in either type of induced cancer.

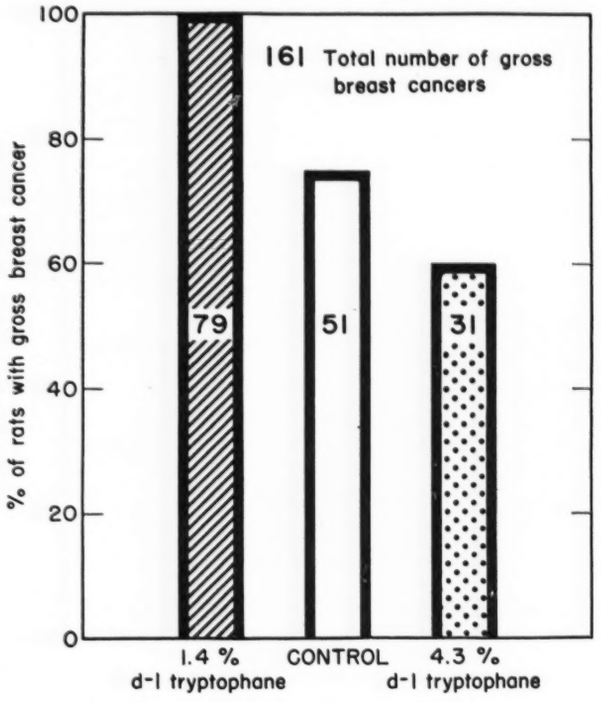

FIG. 2.—The number and per cent of mammary cancers in each group.

The tumors in the three groups were of the same general morphology, with a larger proportion of the more malignant types occurring in the rats on the 1.4 per cent tryptophane diet. The gross, i.e., macroscopic, tumors in the rats of this group included 37 papillary adenocarcinomas, 36 adenocarcinomas and solid type carcinomas, and 6 with varying amounts of squamous-cell cancer. The tumors in the control group comprised 41 papillary adenocarcinomas, 8 solid type and adenocarcinomas, and 2 with areas of squamous-cell cancer. The gross tumors in the rats on the high-tryptophane diet were classified as 29 papillary adenocarcinomas, 1 squamous carcinoma, and 1 early carcinoma. Axillary lymph node metastases were observed in one rat of the control group and lung metastases in one of the rats on Diet 3. One of the rats in the latter group had, in addition, a fibrosarcoma of the stomach, and one of the rats on the high-tryptophane diet had a mixed tumor of the left ovary.

SUMMARY

1. Thirty-nine A×C line 9935 female rats with cholesterol pellets containing 4–6 mg. of diethylstilbestrol implanted in their scapular region were distributed into three groups and placed on isocaloric synthetic rations containing 26 per cent protein with substitutions of 1.4 and 4.3 per cent of DL-tryptophane for tryptophane-free casein hydrolysate in two of the groups.

2. All 39 rats survived the minimum latent period of 163 days, and 30, or 77 per cent, developed 161 macroscopic mammary cancers and a total of 436 independent mammary cancer foci.

3. The largest proportion of tumors (79 macro-

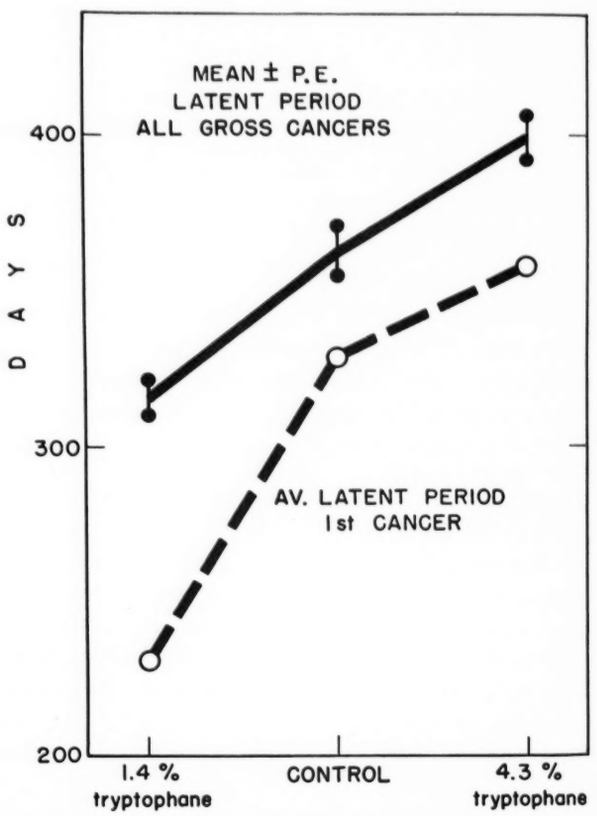

FIG. 3.—The average latent period for the development of first mammary cancers in each group and the mean latent period ± P.E.

scopic or 194 total) was observed in the rats on the diet containing 1.4 per cent DL-tryptophane and the smallest number (31 macroscopic and 109 total) in the rats on the diet containing 4.3 per cent DL-tryptophane.

4. The average latent period of 316 ± 5.7 days for the tumors in rats on the 1.4 per cent tryptophane diet was significantly shorter than that for the tumors in rats on the control diet (363 ± 8.6 days), or the average latent period of 399 ± 7.5 days for the tumors in the rats on the high-tryptophane diets.

5. The addition of approximately 1 per cent of dietary tryptophane appeared to enhance the development of diethylstilbestrol-induced mammary cancer, while the greater increase of 4 per cent in dietary tryptophane inhibited the formation of these tumors. Depletion in body weight or the consequent inanition may explain the latter result.

6. The 161 macroscopic tumors included 107 papillary cyst adenocarcinomas, 44 adenocarcinomas and solid type carcinomas, 9 with varying amounts of squamous-cell cancer and 1 unclassified.

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The Effect of Age on Regeneration of Rat Liver*

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Because cancer is frequently associated with aging, and with the processes of chronic injury and repair, it is important to estimate the type and amount of response to an effective growth stimulus that can occur in aging tissue. Accordingly, we have undertaken a study of the effects of age upon a regenerating tissue.

Rat liver was chosen for this purpose, because it can readily be induced to proliferate by means of partial hepatectomy. During the first 3 days after operation, it grows far faster than most neoplasms. In addition, its restorative capacity can be accurately measured.

It has usually been considered that the ability of tissues to regenerate decreases with age, and, as far as the liver is concerned, this concept has been borne out by the work of previous investigators. Norris, Blanchard, and Povolny (12) found that the rate at which liver mass was restored was far greater in young than in old rats. Marshak and Byron (10), studying the effect of age on mitosis, found that the interval between partial hepatectomy and the maximum mitotic count increased directly with the age of the animal. We have attempted to expand their findings by determining the rate of hepatic cell regeneration in rats of different ages.

METHOD

The present investigation is based upon the fundamental studies on liver regeneration initiated in this laboratory by Brues and his co-workers. Their procedure has been followed, with minor modifications (3).

Three groups of male and female albino rats of accurately known ages were used: (*a*) young rats, 4–6 weeks old, (*b*) adult rats, 4–8 months old, and (*c*) old rats, 21–30 months old (averaging $25\frac{1}{4}$ months). Most of the latter manifested emaciation, loss of hair, inertia, and other changes indicative of senility. The young rats weighed an average of 45 gm. (ranging from 25 to 78 gm.), the adult rats 233 (134–349), and the old rats 268 (170–488) gm.

Rats were starved for approximately 24 hours (18–26) before operation or autopsy. They were anesthetized with ether, and the median and left lateral lobes were removed. These were assumed to constitute 68.4 per cent of the total liver. This value is based upon the data of Brues *et al.* and confirmed by our own determinations on thirteen six-month-old rats, which yielded a mean value of 68.3 per cent ± 2.5. The animals were killed by exsanguination under ether anesthesia at intervals of 30 hours, 3, 7, and 14 days after operation. One set of results was expressed as per cent of original liver mass restored:

$$\text{Per cent regeneration} = \frac{\text{weight of liver at autopsy}}{\text{weight of main lobes} \div 68.4 \text{ per cent}} \times 100.$$

These results were corrected for changes in the body weight of the rat during the period of regeneration. Tissues were fixed in Zenker's fluid and stained with eosin and methylene blue, or in Bouin's fluid and stained with hematoxylin and eosin. From counts of the nuclei of hepatic parenchymal cells in a measured area, in sections of known thickness cut from tissue blocks of known mass and volume, the number of such nuclei in the entire liver was estimated. Such counts were made on normal and regenerating livers, and a second set of results was expressed as per cent of the original number of hepatic nuclei restored.

Water content was estimated by drying small pieces of liver to constant weight in an oven at 95° C. In some instances it was also determined by means of an Abderhalden's drying pistol *in vacuo* at 78° C.

RESULTS

Restoration of liver mass.—The values for per cent restoration of liver mass in each age group are shown in Table 1. Following partial hepatectomy, all our rats consistently lost weight. The return of the two older groups of rats to their initial body weight level was variable and in many instances still incomplete at 14 days. Old rats were slower than adults; the former showed a mean loss of 6.2 per cent of their original weight by the end of the second week, and the latter a mean loss of 3.2 per cent. The young, growing rats, on the other hand,

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had returned to their original weight in 7 days. During the second week they gained tremendously—the increment amounting to an average of 39.3 per cent of their initial weight. For purposes of reasonable comparison between growing and nongrowing animals, a correction for changes in body weight during the period of regeneration seemed necessary. In order to treat the data uniformly, the correction was applied to the nongrowing as well as to the growing rats. Both uncorrected and corrected values are shown in Table 1. The correction caused no important alteration in the two older groups of rats, nor in the young rats, except at 14 days after operation, when body growth was very rapid. At the end of the second week of regeneration, correction for the tremendous weight gain in these young rats lowered the value from 157 to 113 per cent of original liver mass restored (Table 1). These results are summarized graphically in Figure 1. The old rats ran very close to the adults in rate of restoration of liver mass; no significant difference was demonstrable. The young rats, however, had a consistently higher rate throughout, finally reaching 113 per cent, as compared to 92 per cent for the adults. There was considerable variation among the individual rats in each group, as shown by the magnitude of the standard deviations (Table 1 and the vertical arrows in Fig. 1 at 7 and 14 days). In spite of this great variability, the difference between the young rats and the others seems to be a significant one, since the two older groups lagged behind throughout. On the basis of Fisher's formula for t (7), P was found to be less than 0.10 for the magnitude of the difference between young rats and adults at 7 days and less than 0.01 at 14 days.

The ratio of liver weight to body weight decreases as the rat grows (Webster *et al.* [16], and our own data in this experiment). Webster found the ratio to be 6.6 per cent for 50-gm. male rats and 6.08 per cent for 75-gm. rats. A correction for this additional factor would serve to exaggerate further the superior capacity of young rats to restore liver mass. The estimated magnitude of this correction in the young rats, based on Webster's data, is shown by the incomplete dotted line in Figure 1.

TABLE 1

PER CENT OF ORIGINAL LIVER MASS RESTORED

The standard deviations* and number of rats in each group are given at intervals after partial hepatectomy.

	Young rats		Adult rats		Old rats		Young	Adult	Old
Time after operation	No. of rats	Per cent restored (uncorr.)	No. of rats	Per cent restored (uncorr.)	No. of rats	Per cent restored (uncorr.)	Per cent restored (corrected for change in body weight)		
16 hrs.			7	36 ± 5	7	41 ±10		38 ± 5	42 ±11
30 hrs.	10	48 ± 8	7	46 ± 8	6	43 ± 7	56 ±10	50 ±10	45 ± 7
3 days	9	72 ±10	11	60 ± 8	7	72 ± 7	78 ± 7	68 ±10	75 ± 9
7 days	27	99 ±22	21	82 ±16	15	80 ±11	98 ±17	90 ±16	86 ±12
14 days	14	157 ±21	16	89 ±13	11	85 ± 9	113 ±18	92 ±14	91 ±12

*Standard deviation: $s = \sqrt{\frac{\Sigma(x-\bar{x})^2}{n-1}}$.

Where x is the individual observation, $\bar{x}$ is the mean and n is the number of rats.

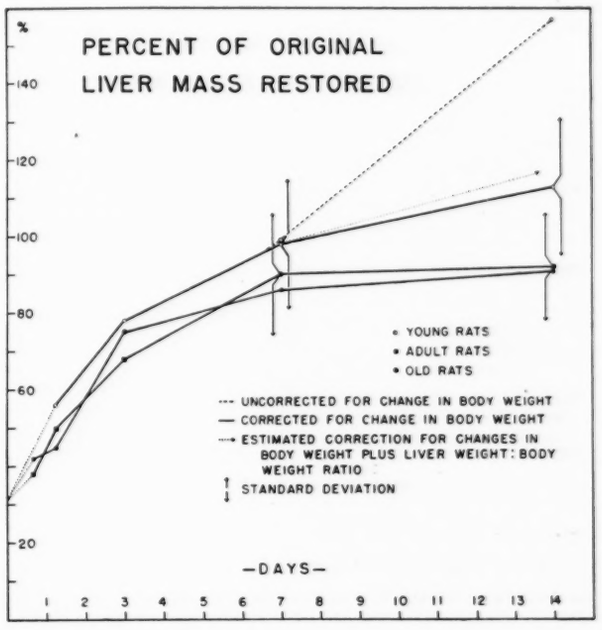

FIG. 1

Restoration of hepatic cells.—The per cent restoration obtained in terms of the number of hepatic nuclei is shown in Table 2. Since endothelial, bile duct, and other cells were not counted, these values represent the ratio of the number of liver cell nuclei in the restored liver to the number in the original liver.

The correction for changes in body weight dur-

ing the period of regeneration has not been applied to these data for two reasons: first, the total number of hepatic cells in a nongrowing rat is assumed to be relatively constant, and, unlike the liver mass, would not be expected to fluctuate with alterations in the animal's body weight and concomitant shifts in hepatic glycogen, fat, or protein storage; second, although the total number of liver cells does increase as the rat grows, this increment is not in direct proportion to the body weight. This second point may be clarified by the following considerations: the livers of the young rats contained approximately half as many hepatic cell nuclei as those of the adults. (We found a mean total of 326×10^6 hepatic parenchymal cell nuclei in the livers of young rats as compared to 651×10^6 in adults and 745×10^6 in old rats). On the other hand, the young rats had more than twice as many of these nuclei in proportion to their body weight (725×10^6/100 gm body weight as compared to 280×10^6 in the adults and 278×10^6 in the old rats). Thus, a simple correction for growth factors on a weight basis would not be justified.

Table 2 shows that, within the limitations of the method, during the first 3 days no significant differences were demonstrable in the rate of replacement of hepatic nuclei. However, by the seventh day the adults and, particularly, the old rats had begun to lag behind the young ones. Since the young rats had just returned to their initial body weight at this point, growth factors did not have to be considered, and the lead they manifested can be considered valid. On the basis of Fisher's formula for t, the differences between young and adult, and adult and old rats, are all statistically significant ($P < 0.01$). By the fourteenth day, the young rats had still further increased their lead, having attained 96 per cent restoration as compared to 74 per cent for the adults and 63 per cent for the old rats. However, since the second postoperative week was a period of very rapid growth for these young animals, 96 per cent is undoubtedly too high a figure and should be corrected downward. Unfortunately, sufficient data are not available for arriving at an estimate of the true value.

TABLE 2

PER CENT OF ORIGINAL NUMBER OF HEPATIC NUCLEI RESTORED

The standard deviation* and number of rats in each group are shown at intervals after partial hepatectomy.

Time after operation	Young rats		Adult rats		Old rats	
	No. of rats	Per cent restored (uncorr.)	No. of rats	Per cent restored (uncorr.)	No. of rats	Per cent restored (uncorr.)
30 hrs.	4	28 ± 4	6	26 ± 3	6	24 ± 7
3 days	6	42 ± 8	10	45 ± 8	5	46 ±11
7 days	16	74 ±18	18	62 ± 7	14	51 ± 9
14 days	13	96 ±15	13	74 ±14	11	63 ± 7

*See footnote Table 1 for standard deviation.

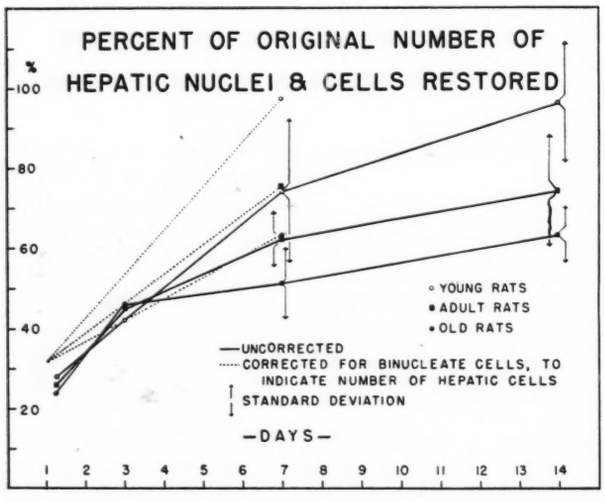

FIG. 2

These results are summarized by the solid lines in Figure 2, which represent rates at which hepatic nuclei are restored to their original numbers in each age group. These do not provide a satisfactory index of the rate of increase of hepatic cells, however, since in the young rats there are about twice as many binucleate cells as in the others.

Table 3 shows the incidence of binucleate cells in liver before and during regeneration. The results, based on random counts of 500–1,000 cells, were found to be quite consistent within each age group. There was a striking decrease in their numbers in all of the regenerating livers, as previously noted by Sulkin (15).

Since the values in Table 3 are derived from counts performed on histological sections, they are only relative. It is obvious that the true numbers of binucleate cells would be considerably higher, because the diameters of single nuclei (6–12 μ) are

greater than the thickness of the slice (6 μ), and, accordingly, many of the pairs will be separated by the sectioning process. Furthermore, the larger the cells, the less chance there is of including both nuclei in a section of uniform thickness; thus, an error is introduced because of variations of cell size, both with age and with the process of regeneration.

62 per cent for adults, and 51 per cent for old rats, they then become 97 per cent, 75 per cent, and 63 per cent, respectively, and are shown by the dotted lines in Figure 2.

With respect to restoration of hepatic cells, then, it may be said that, as in the case of liver mass, the adult rats are inferior to the young in regenerative capacity. In addition, the lag appears

TABLE 3

PER CENT OF BINUCLEATE CELLS

Relative percentages of binucleate cells in resting and regenerating livers of rats in three age groups, showing standard deviations* and number of rats in each group. These figures are based upon counts made on sections cut at 6 μ and are approximately $\frac{1}{3}$ of the true values (St. Aubin and Bucher, unpublished).

	Young rats		Adult rats		Old rats	
Time after operation	No. of rats	Per cent	No. of rats	Per cent	No. of rats	Per cent
0	23	22.4 ± 5.6	25	9.3 ±1.8	21	12.0 ± 2.7
30 hrs.	4	10.9 ± 2.3	5	6.6 ±0.42	5	6.2 ± 1.6
3 days	6	2.4 ± 1.9	8	2.2 ±0.97	6	5.8 ± 1.5
7 days	5	7.8 ± 3.2	6	2.1 ±0.76	4	4.9 ± 1.9
14 days	8	10.4 ± 2.9	9	5.9 ±2.9	7	5.9 ± 1.8

* See footnote Table 1 for standard deviation.

On the basis of an entirely different technic, by which whole liver cells are separated and then examined for the number of nuclei (St. Aubin and Bucher, unpublished), it was found that the actual percentages of binucleate cells were approximately 3 times the relative values shown in Table 3.

These later studies, which are still incomplete, indicated that in resting livers the incidence of binucleate cells was 58 per cent for young rats, 31 per cent for adult rats, and 35 per cent for old rats. The percentages remained unchanged, while no increased mitoses occurred during the first 24 hours after partial hepatectomy; but as mitosis became active they decreased abruptly. The percentages in regenerating liver had dropped to 16 per cent, 10 per cent, and 13 per cent, respectively, after 7 days.

If these percentages are applied to the values for the total number of hepatic nuclei in each liver, the total number of liver cells can readily be calculated. The rate of restoration in terms of actual cells will then be found to increase somewhat in all groups, since binucleate cells are more numerous in resting than in regenerating liver. The young rats, however, will exhibit a far greater increment than the others, so that in terms of cell restoration they will again far surpass both the other groups. To demonstrate the magnitude of this factor, the results have been calculated from average values for all three groups at the seventh day after partial hepatectomy; instead of 74 per cent for young rats,

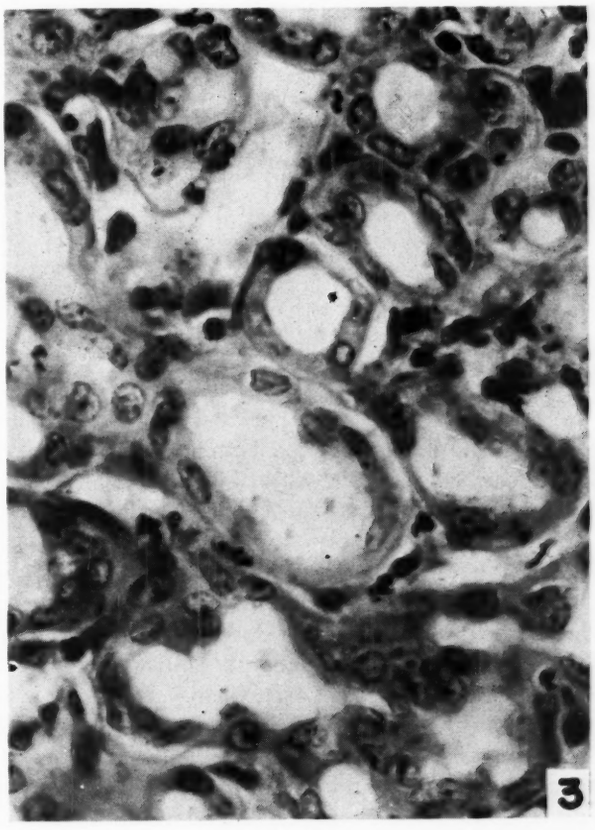

FIG. 3.—Cluster of hyperplastic bile ducts in the liver of a $1\frac{3}{4}$-year-old rat. (Zenker's fix. Eosin-methylene blue stain) ×700.

to be even more pronounced in the old rats. It should be noted that the old rats often had tumors and other conditions tending to debilitate them, as well as a much higher incidence of histological abnormalities in their livers. Nevertheless, the results suggest that advancing age produces at least some degree of additional impairment in the capacity to restore hepatic cells.

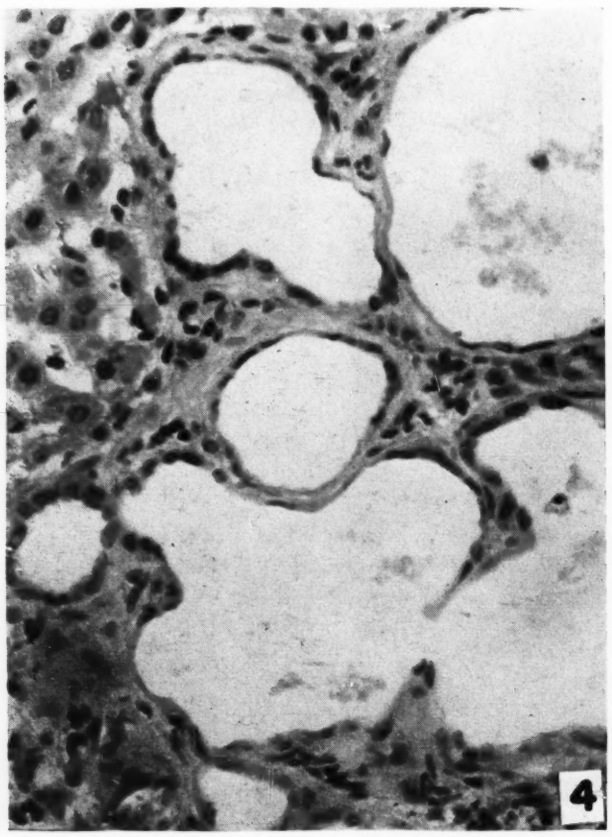

FIG. 4.—Cysts in the liver of a 2-year-old rat. (Zenker's fix. Eosin-methylene blue stain) ×350.

Histological findings.—The resting livers of the old rats showed no definitive characteristics by which they could invariably be recognized. However, certain abnormalities, which were never seen in the young and which were rare and of minor degree in the adult, were found to be relatively common among the old rats. These changes consisted chiefly of hyperplastic clusters of small, round bile ducts, and of single or multiple thin-walled cysts which were surrounded by fibrous stroma (Figs. 3 and 4).

No marked alterations, such as the nuclear inclusions found in senile mice by Andrew, Brown, and Johnson (1), or the syncitial cords seen by MacNider (9) in old dogs, were found to develop in the parenchymal cells with aging. The livers of the young rats, on the other hand, were distinguishable by the smaller size of the parenchymal cells, large numbers of which were binucleate (Figs. 5 and 7).

Occasional aggregations of cells occurred in the periportal areas in rats of all ages—infrequently in the young and often in the old. These infiltrations seemed to consist of leukocytes and mononuclear cells, including macrophages sometimes laden with pigment, fibroblasts, lymphoid cells, etc., in variable numbers and proportions (Fig. 9). Periportal infiltrations of connective tissue cells and lymphocytes were found by Andrew, Brown, and Johnson (1) to be characteristic of senile mouse livers.

The changes taking place in the histological appearance of the liver during regeneration have been carefully described by numerous investigators (13, 6, 3, 4, 2, 15, and many others). There is

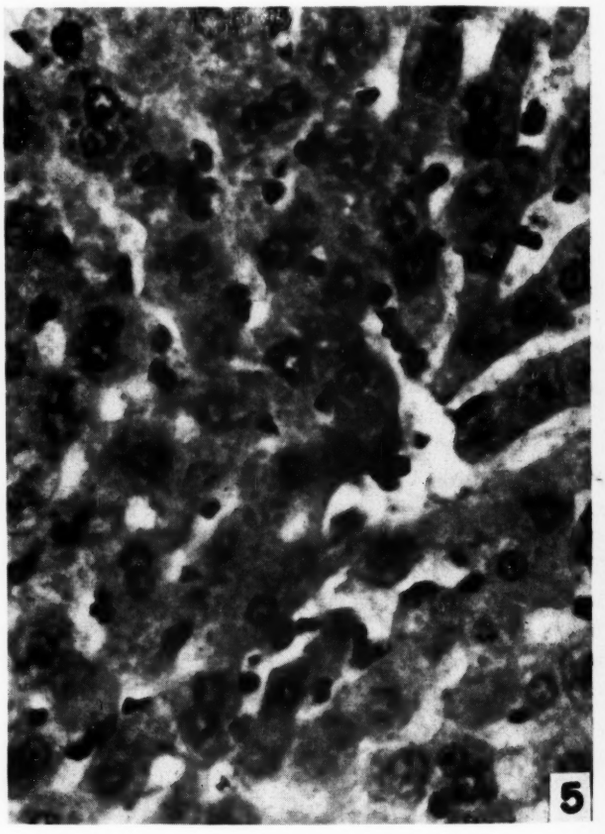

FIG. 5.—Resting liver of a 5-week-old rat. Note small size of cells and nuclei, and large number of binucleate cells. (Zenker's fix. Eosin-methylene blue stain) ×700.

a variable but marked increase in cell and nuclear size, which is apparent late in the first day. The liver cells fill with fat globules, especially in the periportal areas. After 24 hours, mitotic figures appear, scattered throughout the lobules. The increase in cell size and number soon leads to a crowding and irregularity in the alignment of the parenchymal cells that is characteristic of regen-

erating liver, although the fundamental architecture of the lobule is preserved. The basophilia of the liver cell cytoplasm deepens markedly. Binucleate cells, normally common, become scarce. Figures 6 and 8 show such changes in the regenerating liver of a young rat and an old rat, respectively.

As the hyperplasia progresses, the regenerative changes gradually recede, so that, after 2 weeks, they are much harder to observe.

The alterations just described were found to occur in all three age groups in our series, and no essential differences in the over-all pattern of regeneration were observed in most rats. The abnormalities that did occur were found occasionally in the old rats, seldom in the adults, and almost never in the young. They consisted of scattered focal necroses and periportal infiltrations of cells which spread out to invade the surrounding parenchyma (Fig. 10). Tiny focal necroses with collections of wandering cells have occasionally been found in resting livers of supposedly normal rats in our laboratory. These animals were not discarded from our series, because the regenerative rate did not seem to be greatly altered in the rats having these lesions. With one exception they all fell within the same ranges as corresponding rats with histologically normal livers.

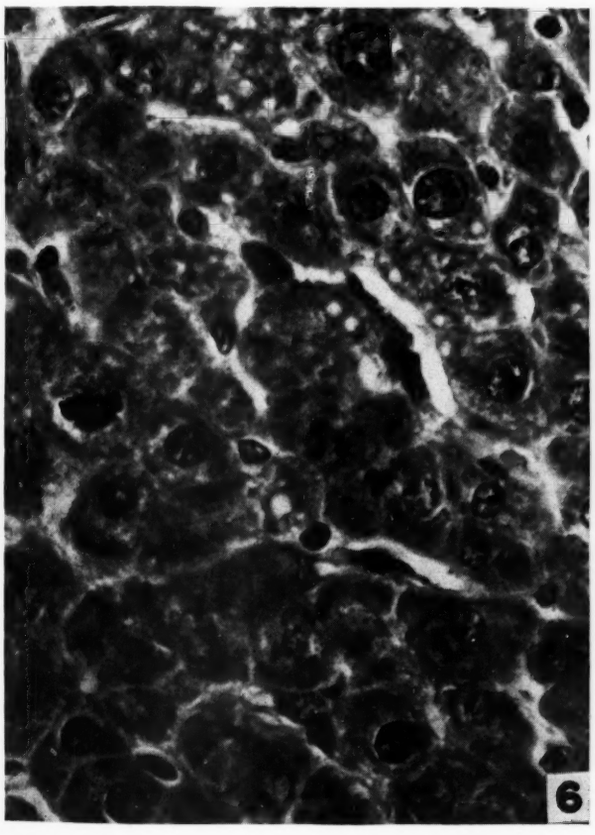

FIG. 6.—Regenerating liver from 5-week-old rat, 3 days after partial hepatectomy. Note cellular and nuclear enlargement and scarcity of binucleate cells. Numerous fat droplets. Mitotic figure at left. (Zenker's fix. Eosin-methylene blue stain) ×700.

The average nuclear diameters which were found in resting liver and at intervals after operation are shown in Table 4. The young rats had definitely smaller nuclei throughout. A marked relative increase in diameter was found in all three groups at 30 hours after operation, and, although somewhat diminished, this enlargement still persisted at 14 days. There was close agreement between the old rats and the adults.

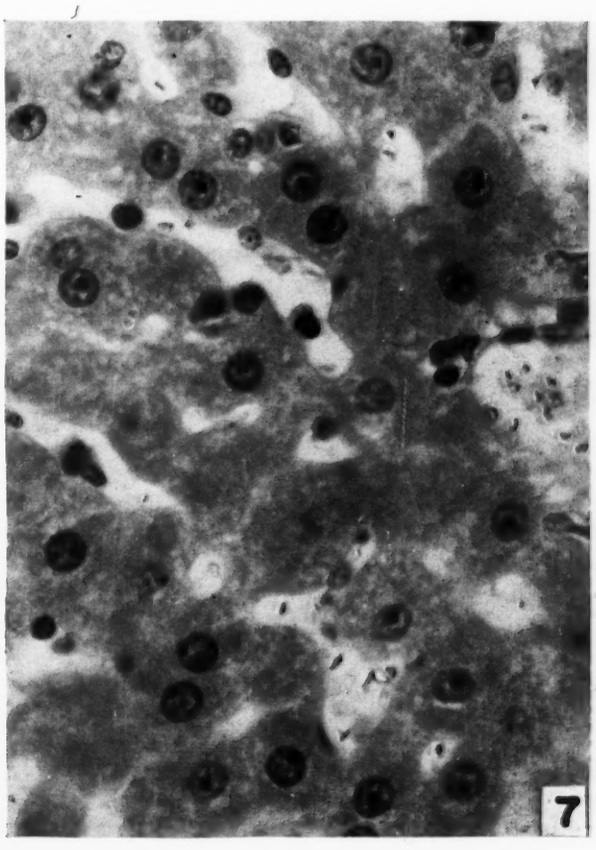

FIG. 7.—Resting liver of a 2½-year-old rat. Note larger size of cells and nuclei as compared with Fig. 5. (Zenker's fix. Eosin-methylene blue stain) ×700.

The average number of hepatic nuclei per cubic millimeter of tissue is shown in Table 5. A marked decrease occurred in all groups during the period of regeneration. According to Stowell (14), who applied Chalkley's method of tissue analysis to this problem, the area occupied by vascular tissue is greatly diminished in regenerating liver. This finding no doubt reflects the crowding-in of newly formed liver cells upon the sinusoids, which is a striking feature of the microscopic picture. At any rate, the decrease in the number of hepatic nuclei

per unit volume of tissue does not represent a mere dilatation of sinusoids, but, rather, it reflects both the increase in cell size and the decrease in binucleate cell content which occur during regeneration. The adult and old rats were closely parallel except at 7 days when the old rats also exhibited the greatest lag in cell restoration, as noted above. By 14 days, they were both approaching normal. The young rats, with smaller cells, many more of which were binucleate, showed higher values throughout. The persistence of cell enlargement in the young rats at 14 days is probably partly a result of the increase in cell size that occurs with body growth.

FIG. 8.—Regenerating liver from 2½-year-old rat 3 days after partial hepatectomy. Note numerous fat globules, marked nuclear enlargement, and mitotic figure. Changes are similar to those shown in Fig. 6. (Zenker's fix. Eosin-methylene blue stain) ×700.

The determinations of wet and dry weight of tissue yielded no data suggesting that changes in water content were of any importance in producing the major differences in regeneration between the three groups.

DISCUSSION

Our results differ in some respects from those of previous investigators. With regard to restoration of liver mass, Norris, Blanchard, and Fovolny (12), using weight of the rat as an index of age, found that young rats (under 100 gm.) responded with an excessive overgrowth, their livers increasing to 145 per cent of the original weight in 7 days. Large rats (over 250 gm.) showed a slow rise to only 95 per cent in 9 days, while intermediate rats reached a peak of 120 per cent at 8 days. No corrections were made for changes in body weight during the period of regeneration. The exceedingly rapid regeneration exhibited by young rats, in their experiment, was also found to occur in our series; this finding persisted, although in a lesser degree, even after our data were corrected for body growth (Fig. 1).

In our colony, where body weight was not found a reliable criterion of age, we did not find significant differences in restoration of liver mass between adult and old rats, although the average age of our old rats exceeded 2 years. It is well known that under highly favorable conditions albino rats can survive for over 3 years, but this is not usual in our climate (5). McCay (11) cites the mean life span of the albino rat as 600 days, and this approximates the usual duration of life in our colony. The old rats used in this present investigation represent

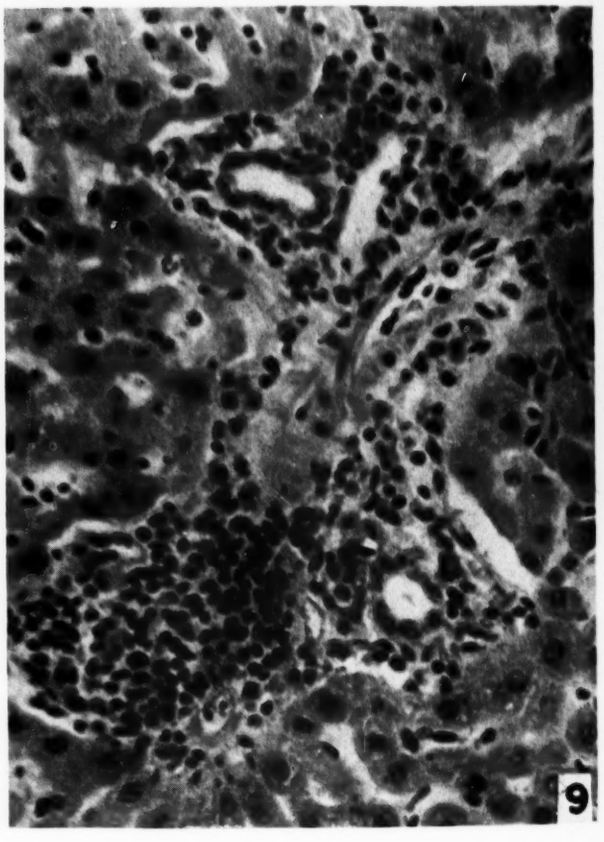

FIG. 9.—Periportal cellular infiltration in the resting liver of a 2⅓-year-old rat. (Zenker's fix. Eosin-methylene blue stain) ×350.

survivors of a much larger group, many of which died, apparently of old age, during the several months preceding the experiment. Hence, it seemed justifiable to consider our rats as old.

With respect to the ability to regenerate new liver cells, our findings indicate a definite decrease with age. Adult rats are greatly inferior to young ones, and old rats even more so (Fig. 2). It is not clear whether the superior capacity of the young animals in this regard is related to the fact that, since they are already in a state of active growth, the added factor of regeneration produces a kind of summation of stimuli, or whether there is merely a progressive decline with age. Possibly, the finding of Marshak and Byron (10) supports the latter idea. They found that age increasingly delayed the initiation of mitosis in regenerating livers. Similarly, Glinos (8), working with tissue cultures of normal rat liver, found a progressive decline in growth capacity with age. On the other hand, he also found that, although resting liver of old rats failed to grow in such cultures, regenerating liver from the same animals grew fully as well as that of young controls. This astonishing increase in proliferative capacity in old rats could still be demonstrated at 45 days after partial hepatectomy, which was many weeks after vigorous mitotic activity had ceased. These findings suggest that certain conditions may arise in a growing liver that enable it to respond more effectively than a resting liver to an additional growth stimulus.

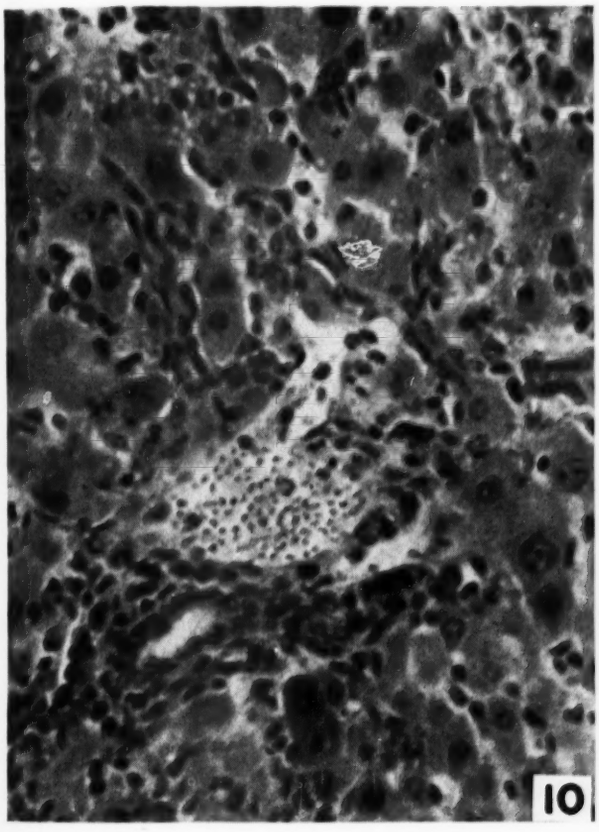

FIG. 10.—Spread of infiltrating cells from periportal areas into surrounding parenchyma. Atypical pattern occasionally seen in certain areas of regenerating livers of old and adult rats. This is from a $2\frac{1}{2}$-year-old rat, 2 weeks after partial hepatectomy. (Zenker's fix. Eosin-methylene blue stain) ×350.

The regenerative capacity may be appraised in various ways. Measurement of restoration of tissue mass does not differentiate between deposition of substances in cells and true growth. The mitotic count provides an index of growth activity at any single moment, but since growth may proceed in waves, the count does not necessarily represent the over-all rate at which a given liver is actually regenerating. The increase in the total number of nuclei during a certain period of time provides more specific information on this latter point; growth is then defined in terms of functional biological units, rather than simple mass. Estimation of regeneration of whole cells is a further refinement of this concept.

The part played by binucleate cells is obscure. They are remarkable for the constancy with which their number characterizes each age group and the

TABLE 4

AVERAGE NUCLEAR DIAMETER (MICRA)

Average nuclear diameters in resting and regenerating livers of rats in three age groups, showing standard deviations* and number of rats in each group.

	YOUNG RATS		ADULT RATS		OLD RATS	
TIME AFTER OPERATION	No. of rats	Nuclear diameter (micra)	No. of rats	Nuclear diameter (micra)	No. of rats	Nuclear diameter (micra)
0	42	6.0 ±0.42	45	7.3 ±0.48	35	7.2 ±0.44
30 hrs.	4	8.0 ±0.28	6	9.0 ±0.59	6	8.9 ±0.92
3 days	6	7.6 ±0.79	10	9.0 ±0.62	5	8.8 ±0.66
7 days	18	7.3 ±0.36	18	8.2 ±0.41	14	9.0 ±1.35
14 days	13	7.6 ±0.57	13	8.4 ±0.45	11	8.4 ±0.72

* See footnote Table 1 for standard deviation.

drastic reduction which occurs in all groups during the process of regeneration. Whatever their significance, it should be noted that the ability to restore liver, by rats of different ages, varies considerably, depending on whether nuclei or cells are regarded as the basic functional unit by which the degree of regeneration is to be evaluated. The trend appears to be in the direction of restoration, not only of the original cell number, but also of the original degree of binuclearity.

SUMMARY AND CONCLUSIONS

Regeneration of the liver following partial hepatectomy has been studied in three groups of rats: young, adult, and old. The livers of the young rats exhibited smaller cells and a much higher degree of binuclearity. Those of the old rats were often indistinguishable from the adults, although they did frequently show certain abnormalities which were rare in the younger animals. During regeneration, the changes in the histological picture were similar in all three groups.

In young rats, regeneration was superimposed upon the normal process of body growth; hence, this group was not strictly comparable to the others. When corrections were made for the various factors associated with growth, however, it seemed that these rats still greatly exceeded the others, in ability both to restore liver mass and to form new cells.

The old rats equaled the adults in ability to regenerate liver mass but lagged behind somewhat in cell production.

ACKNOWLEDGMENTS

We are grateful to Dr. Joseph C. Aub for advice and encouragement in this work, to Mrs. Marie Scott for assistance in performing nuclear counts, and to Mr. Peter Thomson for wet and dry weight determinations on tissues.

TABLE 5

AVERAGE NUMBER OF HEPATIC NUCLEI $\times 10^3$ PER CUBIC MILLIMETER

Average number of hepatic nuclei $\times 10^3$ per cubic millimeter of fixed tissue in resting and regenerating livers of rats in three age groups, showing standard deviations* and number of rats in each group.

	Young rats		Adult rats		Old rats	
Time after operation	No. of rats	Nuclei ($\times 10^3$)	No. of rats	Nuclei ($\times 10^3$)	No. of rats	Nuclei ($\times 10^3$)
0	42	267 ± 62	47	135 ± 32	36	138 ± 21
30 hrs.	4	122 ± 11	6	71 ± 10	6	80 ± 19
3 days	6	169 ± 36	10	87 ± 13	5	89 ± 14
7 days	18	162 ± 44	18	144 ± 18	14	83 ± 19
14 days	13	156 ± 34	13	109 ± 14	11	102 ± 20

* See footnote Table 1 for standard deviation.

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Announcement

The Gordon Research Conferences for 1950, sponsored by the AAAS (formerly known as the Gibson Island Research Conferences), will be held from June 26 to September 1 at the Colby Junior College, New London, New Hampshire. The facilities of the College have been made available for the Conferences.

The Gordon Research Conferences were established to stimulate research in universities, research foundations and industrial laboratories. This purpose is achieved by an informal type of meeting consisting of the scheduled lectures and free discussion groups. Meetings are held in the morning and in the evening, Monday through Friday, with the exception of Friday evening; afternoons are available for recreation, reading, resting, or participation in discussion groups. This type of meeting is a valuable means of disseminating information and ideas which otherwise would not be realized through the normal channels of publication and scientific meetings.

The purpose of the program is not to review the known fields of chemistry, but primarily to bring experts up to date as to the latest developments, to analyze the significance of these developments, and to provoke suggestions as to underlying theories and profitable methods of approach for making new progress.

The first meeting of each Conference is held Monday morning at nine o'clock, Eastern Daylight Saving Time. The morning sessions, through Friday, are scheduled from 9:00 A.M. to 12:00 noon. The second session of each day is held in the evening from 7:30 to 10:00 P.M., Monday through Thursday. There are no Friday evening meetings. Conference members are expected to release their rooms not later than Sunday morning unless they have made a reservation for the next Conference.

Accommodations are available for a limited number of women to attend each Conference, also for wives who wish to accompany their husbands. All such requests should be made at the time of the request for attendance, because these limited accommodations will be assigned in the order that specific requests are received. Children under twelve years of age cannot be accommodated.

Individuals interested in attending the Conference are requested to send in their applications to the Director, as soon as possible. Each applicant must state the institution or company with which he is connected and the type of work in which he is most interested. Attendance at each Conference is limited to 100. Requests for attendance at the Conferences, or for any additional information, should be addressed to W. George Parks, Director, Department of Chemistry, Rhode Island State College, Kingston, Rhode Island. From June 20 to September 1, 1950, mail should be addressed to Colby Junior College, New London, New Hampshire.

The program for the 1950 Cancer Conference will be:

August 28:

GEORGE W. KIDDER. Some Aspects of Comparative Biochemistry of Purines and Pyrimidines.

ALFRED GELLHORN. Experimental Cancer Chemotherapy with Purine and Pyrimidine Derivatives.

DIETRICH BODENSTEIN. Studies of Growth and Differentiation on Embryonic Cells.

L. C. STRONG. Litter Seriation Phenomena in Cancer Susceptibility.

August 29:

E. V. JENSEN and C. HUGGINS. Serum Proteins in Cancer.

STUART W. LIPPINCOTT, HELEN THORNTON, LESTER D. ELLERBROOK, MARSH C. RHEES, C. T. O. FONG, and E. C. STOWELL, JR. Complement Fixation in Animal Neoplasia. A Study of the Factors Involved in the Reaction with the Brown-Pearce Carcinoma.

FREDDY HOMBURGER. Evaluation of Diagnostic Tests for Cancer.

J. K. CLINE. Systematic Study of Some Proposed Cancer Tests.

August 30:

JAMES A. REYNIERS. Germ-free Life and the Cancer Problem.

W. F. DUNNING. Dietary Deficiencies and Excesses in the Economy of Cancer.

JULIUS WHITE and M. BERENBOM. Some Metabolic Studies of Normal and Tumor-bearing Animals with N^{15}.

PAUL C. ZAMECNIK, IVAN D. FRANTZ, JR., and ROBERT B. LOFTFIELD. Labelled Amino Acids in the Study of Normal and Abnormal Growth.

August 31:

W. E. SMITH and D. A. SUNDERLAND. Experimental Studies with Carcinogenic Materials from the Petroleum Industry.

KENNETH M. LYNCH. Asbestos and Cancer of the Lung.

(Speaker and Subject to be announced.)

F. DURAN-REYNALS. Environment and Virus Variations.

September 1:

R. W. RAWSON. What Radio-Iodine Has Taught Us about the Pathologic Physiology of Thyroid Neoplasms.

A. HADDOW. Nitrogen Mustard Carcinogens and Their Site of Action.

W. U. GARDNER, *Chairman*
M. J. SHEAR, *Vice-Chairman*